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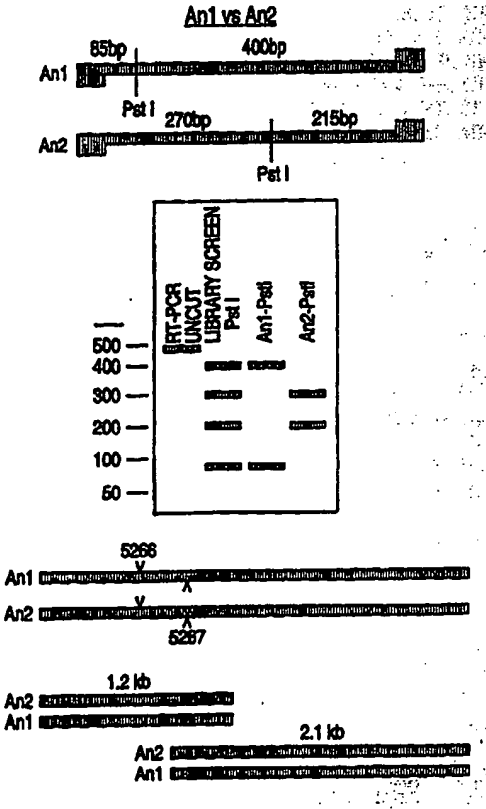
(54) Title: PLANT GENES AFFECTING GIBBERELLIC ACID BIOSYNTHESIS

(57) Abstract

Genes controllong gibberellic biosynthesis are used in genetic engineering to alter plant development. Alterations in the nature or quantity of products of the genes affects plant development. A family of *An* genes in monocots encodes a cyclase involved in the early steps of gibberellic acid (GA) biosynthesis. Members of the family are identified in wheat, barley, sorghum and maize. Two members of the family, the genes *An1* and *An2*, are identified in maize. The *An1* gene is cloned and the function of the gene is characterized. *An2* is isolated and identified by homology to *An1*. Using recombinant genetic technology, GA levels are manipulated. Changes in GA levels alter monocot plant phenotypes, for example, increasing or decreasing height and fertility.



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PLANT GENES AFFECTING GIBBERELLIC ACID BIOSYNTHESIS

BACKGROUND OF THE INVENTION

The present invention relates to genes encoding regulators of gibberellic acid biosynthesis in plants. Plant development is affected by alterations in the nature or quantity of expression products of these genes. A family of *An* genes, found in monocotyledonous plants (monocots), codes for a composition essential for the conversion of GGPP to *ent*-kaurene involved in the early steps of gibberellic acid (GA) biosynthesis. Illustrative members of the family, the genes *Anther ear1 (An1)* and *Anther ear2 (An2)* are identified in maize cloning and functional attributes of the *An1* and *An2* genes are described. *An* genes are also identified in barley, sorghum and wheat by their homology to the *An1* gene of maize.

That GA is important in plant development is illustrated by the correlation between increased vigor in hybrid maize and higher GA levels compared to parental levels, and the greater response of inbreds (compared to hybrids) to exogenously applied GA content (Rood *et al.*, 1988). Further, RFLP analysis points to known GA biosynthetic loci as quantitative trait loci (QTLs) for height in maize hybrids (Beavis *et al.*, 1991), suggesting a role for GA in heterosis. The importance of GA in plant development is further evidenced in the phenotype of GA-deficient mutants of maize, which includes: reduced plant stature, due to shorter internode lengths; shorter broader leaves; less branching of the tassels; and the development of anthers on the normally pistillate ear, resulting in perfect flowers (Emerson and Emerson, 1922).

In maize and probably other plant species, the reduced stature is primarily the result of a decrease in the final length of shoot cells. A reduction in the number of cells per internode is also a factor. Although GA deficiency affects maize shoot and mesocotyl cell length, coleoptile cell lengths are unaffected, suggesting that coleoptile cell extension is independent

of GA. The reduced plant height of GA deficient/responsive mutants of maize is a characteristic common to GA deficient/responsive mutants from a number of plant species including *Arabidopsis*, tomato, rice, pea, and barley. Interestingly, the reduced height phenotype appears to be more responsive to GA levels than the development of anthers on the ear. This is true because, despite the semi-dwarfed to non-dwarfed stature of *An1* mutants, they remain anther-eared.

Gibberellic acid levels also affect fertility in plants. For example, GA can be sprayed directly on plants to affect fertility. The nature of the effect is species specific, that is, in some species excess GA enhances fertility; whereas, in other species, GA reduces fertility. The effect depends on the reproductive mechanics of the species, and on the structure or function affected by GA.

In maize, a monecious plant with diclinous flowers, staminate flowers form on the tassel, while pistillate flowers form on the ear. Maize ears arise from axillary buds. Protuberances develop in an acropetal gradient on the ear that bifurcates-becoming two lobed. However, the diclinous nature of the mature flowers belies the fact that all flowers in the tassel and ear are initially perfect. Very early during their development, differentiation of pistillate and the staminate structures is arrested in the tassel and ear, respectively (Cheng *et al.*, 1983). Flowers, known as florets in maize, are paired in the ear. Each pair arises from bifurcation of a spikelet, with one floret proximal to the ear axis and the other distal. Development of staminate structures in the ear is arrested in both florets, as is development of the pistillate structure in the proximal floret. Thus, the ovule of the distal floret contains the only mature gametophyte found in the ear, and when the enclosed egg and polar nucleus are fertilized, they develop as a kernel. Florets in the anther arise in a similar

fashion, with development of the pistillate structures of both florets arrested very early, while stamens develop in both florets.

Reduced GA levels affect the development of pistils and stamens in maize by arresting development of the stamens in both florets of the ear. This results in a staminate flower in the proximal floret and a mature perfect flower in the distal floret. The development of pistils and stamens in the tassel of GA deficient mutants is delayed, but otherwise is unaffected. Thus, GA is required for the normal arrested development of stamens observed in both florets of the ear. The proximal anthers on ears of GA deficient responsive mutants produce mature pollen that accumulates starch and possesses a germ pore; these are indications of a functional gametophyte. Sexual determination of tassel florets in these mutants appears to be normal, with both florets developing fertile anthers, while the pistillate structures fail to develop. The effect of these mutations on the tassels appears to be limited to reducing branching and causing a poor pollen shed apparently due to failure of the glumes to open.

In maize, tassels and shoots have served as sources for the identification of a number of GA biosynthetic intermediates (Suzuki *et al.*, 1992; Hedden *et al.*, 1982). In addition to being present in shoots, GAs have been shown to be present in root tips of *Pisum* (Coolbaugh, 1985) and in immature seeds of *Pharbitis* (Barendse *et al.*, 1983).

Gibberellic acids are synthesized from the isoprenoid GGPP, beginning with the cyclizations of GGPP to CPP, then CPP to *ent*-kaurene, catalyzed by kaurene synthases A and B (previously kaurene synthetases A and B), respectively (Duncan *et al.*, 1981). Most higher plants are thought to be like maize in that, in maize, *ent*-kaurene is oxidized stepwise to 7-hydroxy-kaurenoic acid, which is converted to the first true gibberellin; GA_{12}-aldehyde (Suzuki *et al.*, 1992). The latter compound then

is oxidized further to an active GA by one of three parallel pathways. In maize the dominant pathway appears to be the early 13-hydroxyl pathway (Hedden *et al.*, 1982), with GA1 being the penultimate, active product, typically present in less than 1 μg/100 gfwt amounts (grows fresh weight of tissue) (Fujioka *et al.*, 1988).

The biosynthetic block in four of the five documented GA-deficient mutants of maize has been predicted by measuring accumulation of endogenous GA biosynthetic intermediates, and measuring growth responses to, and determining the fate of, intermediates (Fujioka *et al.*, 1988). The precise biosynthetic role of the fifth locus, *An1*, has remained undetermined heretofore. Mutations in *An1* result in a GA-deficient phenotype, curable with applied *ent*-kaurene, which suggested that the *An1* gene product functions in *ent*-kaurene synthesis. However, *An* genes have not been cloned, isolated or sequenced. Therefore, genetic engineering methods for manipulating *An* genes to control plants are not available in the art. The availability of genetic engineering for GA levels would accelerate and enhance previously available classic breeding programs.

Genes have been cloned from maize using the Mutator transposable element family (Mu) to generate gene tagged mutants. Among the genes thus cloned are *a1* (O'Reilly *et al.*, 1985); *bz2* (McLaughlin *et al.*, 1987); *hcf106* (Marteinssen *et al.*, 1989); *hm1* (Johal *et al.*, 1992); *iojap* (Han *et al.*, 1992); *vp1* (McCarty *et al.*, 1989) and *y1* (Buckner *et al.*, 1990). However, the use of the Mu system for cloning is not predictably successful.

SUMMARY OF THE INVENTION

Control of levels of gibberellic acid (GA) in plants by genetic engineering techniques requires identification and isolation of genes whose expression affects the operation of the biosynthetic pathway leading to gibberellic acids. Control of GA levels is a means of controlling plant development.

An aspect of the present invention is to identify,

isolate and characterize a family of genes in monocots that is capable of encoding a product that functions to convert GGPP to *ent*-kaurene in gibberellic acid biosynthesis. Monocot includes sorghum, wheat, maize, barley. The family of genes is defined by a capability to hybridize under conditions of high stringency with the *An1* gene from maize, and therefore is designated *"An"*. The genes of this family encode products that are necessary for the conversion of GGPP to *ent*-kaurene in the biosynthesis of gibberellic acid. Without being bound by theory, it is believed that the product is an isoprenoid cyclase. A representative member of the family is the *Anther ear1* (*An1*) gene from *Zea mays*, which has been isolated, cloned, sequenced and characterized. The *An1* gene is required for the accumulation of normal levels of GA in maize, and is understood to encode *ent*-kaurene synthase A, the enzyme involved in the first committed step of GA biosynthesis. Defective mutations of this gene cause the plants to be dwarfed, anther-eared and late- flowering.

Other members of the family of *An* genes of the present invention were located in barley, wheat and sorghum, by means of the ability of a candidate gene to hybridize with an oligonucleotide probe from a maize *An1* gene nucleotide sequence of the present invention. Part of an *An1* clone was used as a probe. Genomic DNA was extracted from barley, sorghum, and wheat plants. Each genus was analyzed separately. The genomic DNA was digested and separated by gel electrophoresis. The separated DNA was blotted. An *An1* DNA probe was used to search for homologous nucleotide sequences in barley, sorghum and wheat. In addition, a maize *An2* gene is detected in maize. Products of *An2* mutant genes decrease GA levels, although to a lesser degree than effected by the *An1* gene product. A double mutant plant, that is, a plant with a mutation in both *An1* and *An2*, may be characterized by a more severe phenotype than either single mutant, that is, a severe dwarf phenotype.

DNA and RNA gel blot analysis demonstrate *An1* to be a single copy gene. Sequence analysis of a 2.8 kb *An1*-cDNA clone shows homology with plant cyclase genes and a polyprenyl pyrophosphate binding domain. The initial steps in the GA biosynthetic pathway involve binding a polyprenyl pyrophosphorylated substrate, geranylgeranylpyrophosphate, which is converted by cyclization to kaurene, steps for which *an1* plants are defective. Northern analysis of the *An1* transcript indicates that it accumulates in shoots, roots, immature ears and kernels, silks and tassels. The transcript does not accumulate in dark grown shoots, suggesting that light is a regulator of *An1* expression. Expression of *An1* was monitored in a number of *an1* isolates, as has its expression in maize shoots, roots, tassels, silks, pollen, and kernels. Light induction of *An1* transcripts have been demonstrated in seedling shoots.

Cloning GA biosynthetic genes provides recombinant genetic tools leading to a better understanding of the role GA plays in the growth and development of maize. In addition, control over GA levels can be used to manipulate plant development by recombinant DNA technology to specific ends.

An1 is one of five identified genes in maize that are involved in GA biosynthesis. Mutants of all five genes (*An1*, *d1*, *d2*, *d3*, and *d5*) are anther-eared, but *An1* is distinct from the others in that its stature is invariably semi-dwarfed rather than dwarfed. The semi-dwarfed stature appears to result from a redundancy in the maize genome for the *An1* function. Evidence for this redundancy comes from *an1-bz2-6923*, a deletion mutant that lacks the *An1* gene yet accumulates *ent*-kaurene, a downstream product of *An1* activity. Further support for redundancy comes from low stringency Southern analysis of *an1-bz2-6923* DNA which demonstrates the presence of sequences with some homology to *An1*.

One of these sequences is identified as the *An2* gene, the existence of which was not suspected from the classical

breeding experiments which identified the other GA biosynthetic maize genes.

The *An1* gene product is involved in kaurene synthesis, early in the gibberellic acid (GA) biosynthetic pathway. Thus, the loss of *An1* function results in a GA-deficient phenotype that causes altered development including reduced plant height and the development of perfect flowers on normally pistillate ears. An *An1* allele was generated by Mutator induced mutagenesis, and the gene was cloned using a DNA fragment that is common to both Mu1 and Mu2 as a mutant gene probe.

The *An1* gene was cloned from maize using a mutant fragment as a gene probe. In a tagged *An1* isolate, *an1-891339*, Mu2 is inserted in the coding region of the *An1* gene. This results in a GA-deficient phenotype. The identity of the *An1* clone was confirmed by a comparison of the predicted amino acid sequence with that of a *GA1* gene from *Arabidopsis* (See PCT patent application WO/9316096). The two genes are 47% identical and 68% similar (GCG package, Genetics Computer, Inc., University of Wisconsin) at the amino acid level, suggesting that they have a common function.

An1 contains a polyprenylpyrophosphorylase binding domain and shares homology in this region with other plant cyclase genes. Southern analysis of a deletion mutant, *an1-bz2-6923*, demonstrated that the *An1* coding region lies entirely within the deletion. But the deletion mutant accumulates kaurene, indicating that *An1* function is partially supplemented by an additional activity. In fact, low stringency Southern analysis of deletion mutant DNA demonstrates the presence of DNA sequences homologous to *An1*, for example, the *An2* gene, which was isolated by the RT-PCR method. Therefore, it is likely that the semi-dwarfed stature of *An1* mutants, as opposed to the dwarfed stature of the other GA-deficient mutants in maize, is based on redundancy in this step of the GA-biosynthetic pathway. A double

mutant, with deficiency in GA levels effected by more than one gene, may show a more severe phenotype than a single mutant.

Antibodies have been prepared to the *An1* gene product. The antibodies coupled with *in vivo* and *in vitro* assays of kaurene synthase A and B activity from *An1* constructs cloned into *E. coli* expression vectors allow the *An1* gene product to be tested for kaurene synthase A and B activity. Complexes were formed with kaurene synthase A and the *An1* clone gene product.

The identity of a second gene product that catalyzes the first committed step in the synthesis of the plant hormone gibberellic acid has been determined through the use of oligonucleotide primers derived from the *An1* sequence. Oligonucleotides homologous to the *An1* nucleotide sequence were generated and used to synthesize a 485 bp RT-PCR fragment that is highly homologous to but distinct from *An1*, as evidenced by a restriction site analysis of a corresponding nucleotide stretch in *An1*. This fragment has been designated as *An2*. The resulting 485 bp RT-PCR product is used to derive *An2* specific primers. These primers are used to isolate full length cDNAs of *An2* and to determine its mRNA sequence.

Changes in plant developmental activity and yield have been accomplished in the past via conventional breeding, which requires an entire genome to be recombined, rather than a single gene or selected set of genes, and which is limited to natural genetic variability rather than being amenable to genetic engineering. The family of genes provided by the present invention permits engineered placement of such genes in a uniform background, for better control of plant developmental aspects such as stature and fertility, and manipulation of the genes *per se* to achieve specific plant breeding objectives. For example, adding *An* genes to a plant to increase GA levels or adding an *antisense* molecule to decrease GA levels.

Definitions

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

In eukaryotes, RNA polymerase II catalyzes the transcription of a structural gene to produce mRNA. A DNA molecule can be designed to contain an RNA polymerase II template in which the RNA transcript has a sequence that is complementary to that of a specific mRNA. The RNA transcript is termed an **antisense RNA** and a DNA sequence that encodes the antisense RNA is termed an **antisense gene**. Antisense RNA molecules are capable of binding to mRNA molecules, resulting in an inhibition of mRNA translation.

A **cloning vector** is a DNA molecule, such as a plasmid, cosmid, or bacteriophage that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

Exogenous denotes some item that is foreign to its surroundings, and particularly applies here to a class of genetic constructs that is not found in the normal genetic complement of the host plant. Thus, in the present invention an exogenous construct used to produce a plant via transformation includes an operative promoter and an isolated DNA molecule having a nucleotide sequence of a member of the family of genes of the present invention.

An **expression vector** is a DNA molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements, including constitutive or inducible

promoters, tissue-specific regulatory elements and enhancers. Such a gene is said to be "operably linked to" the regulatory elements.

Heterologous is a modifier indicated a source that is different. For example, a **heterologous promoter** used with a structural gene of the present invention is a promoter that is different from that of the structural gene.

An **isolated DNA molecule** is a fragment of DNA that is not integrated in the genomic DNA of an organism. For example, the nucleotide sequence of the *An1* gene is a DNA fragment that has been separated from the genomic DNA of a maize plant. Another example of an isolated DNA molecule is a chemically-synthesized DNA molecule that is not integrated in the genomic DNA of an organism.

Isolates are mutant plants derived from independent sources.

A **recombinant host** may be any prokaryotic or eukaryotic cell that contains either a cloning vector or expression vector. This term also includes those prokaryotic or eukaryotic cells that have been genetically engineered to contain the cloned gene(s) in the chromosome or genome of the host cell.

RT-PCR is a method known to those of skill in the art. Components used herein for RT-PCR were obtained from GIBCO-BRL, Garthersburg, Md. The manufacturer's instructions were followed.

Two nucleic acid molecules are considered to have a **substantial sequence similarity** if their nucleotide sequences share a similarity of at least 50%. Sequence similarity determinations can be performed, for example, using the FASTA program (Genetics Computer Group; Madison, WI). Alternatively, sequence similarity determinations can be performed using BLASTP (Basic Local Alignment Search Tool) of the Experimental GENIFO(R) BLAST Network Service. See Altschul *et al.*, *J. Mol. Biol.* *215*:403 (1990). Also, see Pasternak *et al.*, "Sequence Similarity Searches, Multiple Sequence

Alignments, and Molecular Tree Building," in Methods in Plant Molecular Biology and Biotechnology, Glick *et al.* (eds.), pages 251-267 (CRC Press, 1993).

A **suitable promoter** is a promoter that controls gene expression in cells that are to be altered developmentally by the manipulation of genes controlling biosynthesis of GA.

A **transgenic plant** is a plant having one or more plant cells that contain an expression vector.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A is a schematic representation of the GA biosynthesis steps and **FIGURE 1B** focuses on steps catalyzed by kaurene synthetase A and B.

FIGURE 2 is an amino acid sequence comparison between gene products of the maize *An1* gene (top) and an *Arabidopsis* gene, GA1 (bottom).

FIGURE 3 A and B is the cDNA sequence of the *An1* gene isolated from maize (Gen Bank accession number L37750).

FIGURE 4 illustrates the role of gibberellic acid in maturity of maize by reference to a comparison of days required to maturity for *an1-bz2-6923* and its wild-type siblings. GDUSHD is heat units to pollen shed, 25 units ~ 1 day.

FIGURE 5 is a plasmid map of DP6464.

FIGURE 6 is a cDNA sequence of an *An2* gene isolated from maize aligned with a fragment of a corresponding segment of the *An1* gene nucleotide sequence illustrated in FIGURE 3.

FIGURE 7 is a restriction site map of the corresponding nucleotide sequences of *An1* and *An2* according to FIGURE 6.

FIGURE 8 A and B is a nucleotide sequence of the promoter of the *An2* nucleotide sequence of FIGURE 3. Position 7 on Figure 8A the beginning of the promoter; position 2075 on Figure 8B the promoter and position 2076 is the transcription start site of *An1*.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Gibberellic acid (GA) levels are important factors in plant development. Control of GA levels by genetic engineering technology allows alteration of plant phenotypes such as fertility and size. Identification and isolation of genes controlling the biosynthesis of GA, are required for this effort. A family of genes have been identified that is capable of encoding a product that is necessary for the conversion of GGPP to *ent*-kaurene in the biosynthesis of gibberellic acid. the product is consistent in structure with a cyclase. Members of this gene family hybridize with the *An1* gene under conditions of high stringency. These genes also encode products that are the functional equivalent of the sequence in FIGURE 2 within the box. FIGURE 2 shows the correlation between the predicted amino acid sequence of *An1* (top) and that of *GA1* (bottom).

Steps catalyzed by kaurene synthase are as follows: Two rings are closed in the conversion of GGPP to CPP by kaurene synthase A. The third ring is closed, the pyrophosphate group is cleaved, and a carbon-carbon bond is broken and reformed at a nearby site as CPP is converted to *ent*-kaurene by kaurene synthase B (FIGURE 1B).

Also as noted, *An1* is one of five identified genes in maize that are involved in GA biosynthesis. The *An1*, *d1*, *d2*, *d3*, and *d5* mutants of maize compose a class of recessive mutants that are GA deficient and GA responsive. They all appear to be defective in some step of the GA biosynthetic pathway, and they share a number of phenotypes, including reduced stature and the development of anthers on the normally pistillate ear.

Within this class of mutants there are two distinct groups relative to stature. Alleles of *d1*, *d2*, *d3*, and *d5* are typically severe dwarfs, exhibiting an 80% or greater reduction in final plant height. In contrast, alleles of *An1* are less severely dwarfed, typically semi-dwarfed, and in some cases there is no reduction in their

final height. The severity of reduction in shoot height for both groups is also reflected in the degree of reduction in their leaf lengths. For the entire class the reduction in height is scorable in both light and dark grown seedlings. In six day-old dark grown *An1* seedlings, the basis of the reduced height lies in the cells of the mesocotyl. Coleoptile cell number is slightly reduced in *An1* seedlings, while the average cell length of coleoptile cells is the same as found in wild-type siblings (Table 1). This is in contrast to the mesocotyl where cell number is reduced by one-half and average cell length is reduced to one-fourth of that observed in wild-type seedlings. Thus, the reduced stature in dark grown seedlings is due primarily to greatly reduced final cell lengths.

Table 1. Comparison of Cell Length and Cell Number in Shoots of Dark Grown Maize Seedlings.

	Length(mm)	Number of Cells	Average Cell Length(mm)
Tall	Sibling		
Coleoptile	18	228	0.08
Mesocotyl	70	294	0.18
Total	88	522	
Dwarf (*An1*)			
Coleoptile	14	171	0.08
Mesocotyl	6	130	0.05
Total	20	301	

Seedlings were grown for six days in total darkness.

The *An1* gene was cloned using transposon tagging. A key advantage for tagging genes with mutator is the 50-fold or greater increase in mutation frequency compared to spontaneous rates. See Walbot, 1992 for a review. Transposon tagging involves using any one of a number of naturally occurring plant transposons -- Mu, Ac, Spm and the like -- to create a "molecular tag" to recover the mutated gene. Although it has been used before, the transposon-tagging approach to recovering a gene of interest is unpredictable, is plagued by a low mutation

frequency, and is very difficult technically. First, the genetic stocks have to be screened phenotypically for mutants of interest. There is no way to direct the transposon to a particular gene or to produce a particular phenotype. After a mutant phenotype of interest is found, moreover, it is necessary to determine whether the mutant is actually caused by the insertion of a transposon, because not all mutations are caused by transposable elements. A gene can be isolated by transposon tagging only if a particular transposon has inserted into the gene.

Each transposon system has major advantages and disadvantages. Ac and Spm, for example, occur in lower copy number per genome than Mu and therefore, promote a lower frequency of mutations. Because both of these elements excise from the germline at a higher frequency than Mu, however, it is possible to use the powerful genetic tool of looking for a reversion of the mutant phenotype as a result of excision of the element from the germline. This provides very strong evidence that a particular mutant was caused by the transposon insertion. Mu has the advantage of having a high copy number, so the frequency of causing mutations is higher (up to 10-100X higher than the background mutation rate.) Because the germline excision frequency is very low (~1 in 10,000), however, standard tests for reversion are not practical. Other, labor-intensive means need to be used to prove that the gene is tagged by the transposon. Those methods are molecular detection methods which involve isolating DNA from the mutant plants of interest, and probing the DNA for the presence of a Mu element which co-segregates with the mutant phenotype. With Mu this is particularly difficult, because there are many copies of Mu per genome - in fact, some genomes have over 200 copies (Walbot and Warren, 1988).

For the present invention, co-segregation of an *an1-891339* phenotype and Mu2 containing restriction fragments was demonstrated by Southern Blot Analysis. DNA from

individual homozygous F2 dwarfed *an1-891339* siblings was analyzed to determine linkage between the mutation and a Mu element. DNA was restricted with SstI, and the blot was probed with an internal Mu2-DNA fragment. A Mu2 containing restriction fragment of 5.7 kb, common to all tested individuals, was identified. This Mu2 containing restriction fragment was cloned into a lambda vector. DNA gel blot analysis of a restriction digest of the clone was performed. Double digests of the cloned fragment was in Lane 2 (SstI and HindIII) and Lane 3 (SstI and XbaI).

Flanking sequence DNA was identified, and a 2.6 kb flanking sequence fragment (g2.6Xba) was subcloned and used as a probe. Southern blot analysis of the deletion mutant *(an1-bz2-6923)* was performed as follows: Southern blots of SstI digested genomic DNA of the deletion mutant and wild-type sibling DNA were analyzed. A blot probed with genomic flanking sequence subclone g2.6Xba showed deletion mutant plants lack DNA homologous to g2.6Xba. Using g2.6Xba as a probe, a 2.8-kb cDNA clone was recovered from a maize cDNA library. This cDNA appears to represent full-length mRNA based on RNA gel blot analysis: the primary product is a homologous transcript of a 2.8-kb. The cDNA contains an open reading frame of 2.5 kb or 823 amino acids, as illustrated in FIGURE 3.

A sequence comparison of maize *An1* and *Arabidopsis* *GA1* showed the complete predicted amino acid sequences of *An1* and *GA1* are similar. Overall identity is 47%, similarity 68% (GCG package, Genetics Computer, Inc., University of Wisconsin). A putative polyprenylpyrophosphorylate binding domain is indicated with a box (FIGURE 2).

The homology between predicted amino acid sequences of maize *An1* and *Arabidopsis* *GA1* points to a common function for these genes. Their overall identity of 47% (68% similarity) is striking, but is even stronger in an internal 300 amino acid segment that is 68% identical

(94% similar). As to the putative polyprenyl-pyrophosphate binding domain within this segment, *An1* and *GA1* share 100% similarity. Other sequenced plant genes that use polyprenylpyrophosphorylated substrates (geranyl-, farnysyl-and geranylgeranyl-pyrophosphate) also share significant homology with *An1* in this domain (Facchini *et al.*, 1992), but much less overall homology with *An1* (20 to 25% identity). These sequence homologies clearly indicate that *An1* encodes a cyclase which functions in the conversion of GGPP to *ent*-kaurene.

While highly homologous to *GA1*, it is important to note that *An1* is distinct from *GA1* in its amino (only 11% identical for first 100 amino acids) and carboxyl terminus (only 18% identical for the last 283 amino acids). Also, the amino terminus of *An1* has characteristics expected of a chloroplast targeting sequence including a net positive charge (12 of 43 amino acids are basic while only two are acidic). In addition, the *An1* amino terminus also has a greater than 50% similarity to the amino terminus of an aspartate aminotransferase cDNA clone from rice (Gene Bank Source D16340). Aspartate aminotransferase has many isoforms, at least one of which is located in the chloroplast (Matthews *et al.*, 1993). This suggests that the amino terminus of *An1* serves as a chloroplast-targeting sequence. Support for a chloroplastic localization of kaurene synthesis comes from the demonstration that cell free assays of purified chloroplasts synthesize kaurene (Simcox *et al.*, 1975). If *An1* and *GA1* code for the same chloroplast targeted activity their targeting sequences are distinct. The low homology between *An1* and *GA1* in their carboxyl termini may be functionally important. While a number of plant cyclase activities share a conserved polyprenylpyrophosphate binding domain, they act on distinct substrates and cyclize by distinct mechanisms. The basis for these differences is not obvious from an examination of the primary amino acid sequences.

Southern blot analysis using high and low stringency was performed. Southern blots of homozygous deletion *an1-bz2-6923* and wild-type sibling DNA compared from high (at a temperature of 65°C) and low (at a temperature of 25°C) stringency washes were compared. Genomic DNAs were digested with BamHI. The probe was *An1*-cDNA. Therefore, at high stringency, probe DNA, hybridizes only to wheat and tall sibling DNA, whereas, at low stringency, hybridization occurs with deletion mutant maize. A related sequence is likely in wheat.

Northern blot analysis shows *An1* transcript accumulation. Northern blots from total RNA preparations were probed with *An1*-cDNA. Tissues analyzed were:

(A) shoots and roots of light and dark grown seedlings; and

(B) reproductive structures.

The blot revealed *An1* transcript accumulation in all tissues and an enhancement of accumulation in light grown shoots.

Since GA plays important developmental roles, its control is a useful avenue to altering development for specific purposes. The *an1-bz2-6923* allele of *An1* is consistent with a robust plant which demonstrates little or no reduction in plant height or leaf length compared to wild-type siblings. Despite its similarity in growth, the average first day of pollen shed in this mutant is delayed, in the example shown this delay is 5 days (FIGURE 4). This demonstrates that lowering GA levels reduces time-to- maturity in maize, possibly by shortening the time required between germination and floral initiation.

A comparison of days required to maturity for *an1-bz2-6923* and its wild-type siblings is shown in FIGURE 4 as a plot of the height of wild-type siblings and *an1-bz2-6923* mutants versus GDUSHD (heat units to pollen shed, 25 units ≈ 1 day). Although no difference in final height exists, there is an average of 200 GDUSHDs delay for the mutant plants. Shortened time to maturity is an

advantage in some growing zones (climates); whereas, increased time to maturity is an advantage in other growth zones. Therefore, the ability to manipulate GA levels by recombinant techniques is advantageous for developing commercial monocots. Isolation of genes such as *An1* provides some of the tools needed for this endeavor. The *An1* gene will also be useful to probe for homologous genes in other species. A gene homologous to *An1* was isolated by RT-PCR. Construction of the primers used to generate the 485 bp RT-PCR product was completely dependent upon the previously determined *An1* cDNA sequence as shown in by Bensen *et al.* (1995). Further oligonucleotide primers were generated from the 485 bp RT-PCR product.

Primers that are specific for *An2* are used in a reverse genetics screen. A collection of corn families is used that has a high frequency and, perhaps many mutations. The large number of families is screened in sets of about 50 for gene mutations in areas of interest. PCR primers are defined for the mutator elements. Primers from the *An2* fragment are matched to those in the families to detect specific families that have Mu inserted near the tested primer product of interest. Such families are then used for various breeding crosses. Plant families selected by this screen have Mu insertions in the *An2* gene. Seed from progeny F_2 plant families are grown. No dwarfing phenotype is likely for these families, because *An2* mutants only have 20% reduced levels of GA. However, crosses between these families and *An1* mutant plants produce double mutants which are severely dwarfed, because both a 20% and an 80% decrease are combined. Alternatively, if *An1* and *An2* are different, complementation occurs.

The present invention is illustrated in further detail in the following examples. These examples are included for explanatory purposes and should not be considered to limit the invention.

EXAMPLE 1

Cloning the *An1* Gene

Reports in the literature suggest that GA levels may be a partial cause of heterosis. To develop transgenic tools for improving yield in crop plants using genes affecting GA synthesis, a goal was to clone genes which encode enzymes of the GA biosynthetic pathway.

Several GA-deficient mutants of maize had been described (d1, d2, d3, d5, *An1*) which were associated with a dwarf stature and andromonoecious flowering (perfect flowers on the ear). If these mutations actually occurred in the genes directly coding for GA biosynthetic enzymes, it was difficult to envision how to identify and isolate the genes without having to purify the as yet uncharacterized enzymes in the GA pathway. One possible approach was to use transposon tagging, which had been successfully used in some cases to tag and isolate genes (Walbot, 1992). But dwarfs are very rare and, moreover, no known transposon- induced alleles had previously been reported for any dwarf mutants. An anther ear (*An1*) mutation segregating in a Mu-containing maize line was obtained from Patrick Schnable (Iowa State University), and experiments were carried out to determine whether a transposable element could be found associated with the mutant gene. The likelihood of this was questionable, however, because such transposon-tagged dwarf mutants had never been identified before.

The employed mutant-detection method involved isolating DNA from the mutant plants of interest and then probing the DNA for the presence of a Mu element which co-segregates with the mutant phenotype. This was particularly difficult because there are many copies of Mu per genome; in fact, some genomes have over 200 copies (Walbot and Warren, 1988).

In order to reduce the extremely large number of Mu-hybridizing bands, it was first necessary to make repeated crosses to plants that inactivated and diluted

out most of the Mu elements. It was also necessary for the *An1* mutant gene search to use Southern blots to probe genomic DNA separately with a DNA fragment that is unique to each of nine distinct Mu families. Even then, the number of copies per Mu family is around 25, making it very difficult to identify one hybridizing band in the blot that co-segregates with the Mu element used as probe. In doing such a DNA screen for *An1*, it was necessary to prepare DNA from 50 different individual plants and probe each of those samples in a Southern blot with each of the Mu-specific probes, Mu1, Mu2 and Mu3, that are characteristic of the sub-family.

After a Mu-tagged, co-segregating restriction fragment was found, the fragment was isolated by cloning and sequenced to identify the location of the Mu insertion. The flanking regions were also sequenced, to locate the structural gene of interest. For a gene like *An1*, not identified or isolated previously and, hence, of unknown sequence, it can be very difficult to determine the exact limits of the gene and even to prove that the clone contains the mutant gene of interest. As Walbot indicates in her 1992 review of strategies for mutagenesis and gene cloning using transposon tagging, identification of a co-segregating band is not straightforward. Moreover, identification of such a band is not proof that the band in question defines the gene of interest.

A family with a phenotype characteristic of GA deficiency was observed to segregate as a simple recessive trait in an active Mu line. The mutation was shown to be allelic with *An1*, and was identified as *an1-891339*.

Southern analysis of *SstI*-restricted genomic DNA from *an1-891339* and its wild type siblings identified a Mu2-containing restriction fragment, of approximately 5.4 kb, which co-segregated with the mutation. This fragment was eluted from a preparative agarose gel, cloned into a bacteriophage lambda vector and plaque purified using a Mu2 internal fragment as a probe. Analysis of the cloned

fragment, by restriction with XbaI or HindIII, identified fragments of flanking sequence DNA. A 2.6kb XbaI flanking sequence fragment (g2.6Xba) was subcloned into a plasmid and used as a probe for Southerns and screening maize cDNA libraries. Southern analysis of maize genomic DNA demonstrated that g2.6Xba was single copy DNA.

Using g2.6Xba as a probe, a number of cDNA clones were selected from maize cDNA libraries, demonstrating that g2.6Xba lies in a transcribed region of the genome. The frequency of positive clones in each of two amplified libraries was 8 per 360,000 plaques. The longest of the cDNAs, 2.8kb, was subcloned into a plasmid and sequenced. This cDNA appears to represent full length mRNA.

Comparing cDNA and *An1* genomic DNA sequence identifies a number of exons. The comparison also demonstrates that the Mu2 element causing the mutation is inserted within or at the border of an intron, 1.6 kbp from the carboxyl terminal of the transcript and 900 bp from the amino terminal.

It was necessary to take several approaches to confirm the identity of the putative clone of the *An1* gene. Tight linkage between the clone and the gene needed to be established by testing to show that the clone did not hybridize to DNA from a known genetic deletion mutant of *An1*. This evidence placed the clone to within a few map units (4 centimorgans) of the genetic locus for *An1*, based on the resolution of this mapping experiment. That distance corresponds to ~8.4 Mb x 10^6 bp, so it is possible the clone could have been located as far away as 8.4 mb from the genetic locus for *An1*.

The next step was to isolate and sequence a cDNA clone. To do this, it was necessary to determine where the putative *An1* gene was expressed so that a cDNA library could be created that was likely to contain the gene. Because the size of the mRNA was known to be quite large (~3 kb), recovery of a full-length clone was very difficult.

The first clone was only 2.5 kb in size, so it was necessary to screen a second library to recover a longer clone of 2.8 kb. The sequence of the cDNA showed ~40% similarity in only one region of the clone to an isoprenoid cyclase type of binding region, based on other known cyclase-type genes.

The biochemical function of *An1* is known to be required for kaurene accumulation and is likely the cyclase which converts GGPP to CPP. This is known to be the first committed step in GA biosynthesis (kaurene synthase A).

Homology with other cyclases was consistent with one of the possible functions for the *An1* gene product. The homology that was seen was very limited and far less than the overall homology typically seen among cyclases, so only tentative conclusions could be drawn as to the identity of the isolated gene. Therefore, additional evidence had to be obtained from other technical approaches.

Peptides were synthesized that corresponded to predicted antigenic domains of the protein which was encoded by the clone. Antibodies were raised against several peptides. Only 2 of the 4 antibody preparations were usable. Some of the antibodies were shown to precipitate the GGPP-to-CPP cyclase activity of cucurbit endosperm extracts, providing additional evidence to support the possibility that the isolated gene was *An1*. Finally, a comparison of amino acid sequence between our clone and a GA1 clone from *Arabidopsis* revealed significant homology throughout the length of the protein. GA1 has been shown to encode the GGPP-to-CPP cyclase (Tai-Ping Sun *et al.*, personal communication).

These data provide a convincing case that *An1* was cloned, but clearly, the process was a difficult and uncertain one. Although transposon tagging made it possible to clone the *An1* gene, success was far from predictable.

The efficiency of obtaining an insertional mutant depends on a variety of factors, including the activity phase of the autonomous element(s), the number of mobile elements, the location of the elements and the susceptibility of the target locus (Walbot, 1992). As Walbot states in her review, "Although not often reported, some targeted mutagenesis screens fail completely, despite reasonable progeny sizes". Table 2 in her review indicates a number of examples where attempts to target specific genes by transposon insertion have failed. Based on the previous failure to identify any dwarf mutants which were transposon-tagged, it was not unreasonable to assume that the target locus for genes in the GA pathway might not be susceptible to tagging. Therefore, it was very uncertain that the *An1* mutant from the Mu genetic stocks was in fact tagged by Mu. However, the *An1* gene has been cloned, as shown herein.

EXAMPLE 2

Basis for Semi-Dwarfed Nature of *An1* Plants

As described previously, *An1* is unlike the other GA deficient/responsive mutants of maize in that it is a semi-dwarf. This is true of all four isolates of *An1* examined. *An1* plants respond to the application of a number of GA biosynthetic intermediates, including *ent*-kaurene. Since GA biosynthesis is initiated by the conversion of GGPP to CPP, followed by the conversion of CPP to *ent*-kaurene, *An1* appears to be deficient in the conversion of GGPP to *ent*-kaurene.

Probing *an1-bz2-6923* DNA on a Southern blot with either g2.6Xba or full length *An1*-cDNA resulted in no detectable hybridization of probe. Similar results were observed on northern blots of deletion mutant RNA. This indicates that the transcript of the *An1* gene lies entirely within the deletion and is therefore not present in *an1-bz2-6923* plants.

It would be expected, therefore, that this mutant would be absolutely defective in *ent*-kaurene synthesis.

Yet light- grown *an1-bz2-6923* seedlings accumulate *ent*-kaurene *in vivo*, albeit at a much reduced rate (20%) compared to their wild-type siblings (Table 2). This activity is attributed to the *An2* gene product, a non-*An1* activity that supplements *An1* production of *ent*-kaurene. The supplementary activity is thought not to be unique to maize. A deletion mutant of *Arabidopsis*, *GA1-3*, also is expected to be devoid of *ent*-kaurene, since the *GA1* coding region is entirely deleted (Tai-Ping Sun *et al.*, 1992). However, *GA1-3* plants convert GGPP to CPP and CPP to *ent*-kaurene in cell-free extracts of siliques. Notably, there are a number of *GA1* isolates that demonstrate a uniform but variable reduction in plant height similar to that observed for the *An1* isolates in maize. The accumulation of *ent*-kaurene is not observed in maize *d5* mutants, however. The *d5* mutant is believed to be defective in kaurene synthetase B as is the *GA2* mutant of *Arabidopsis* which has A, but no B activity in cell free extracts from immature siliques. When the stringency of Southerns is lowered for blots of restricted *an1-bz2-6923* DNA, by altering temperatures, bands sharing homology to *An1* can be identified suggesting that homologous sequences provide *An1* functional equivalents.

Thus, the consistent "leaky" or semi-dwarfed phenotype observed for all documented *An1* mutants in maize is likely the result of a redundancy for *An1* function. This redundancy does not exist, or is of little significance, for the kaurene synthetase B-encoding maize *d5* and *Arabidopsis* *GA2* genes, since their block in kaurene synthesis seems complete.

EXAMPLE 3

An1 Transcript Distribution and Expression

Transcription of the *An1* gene in maize occurs in a number of tissues, as demonstrated by northern blots. Vegetative parts of the plant, shoots and roots, contain *An1* mRNA. Reproductive tissues including tassels, developing ears, silks and embryos all contain *An1* mRNA.

Interestingly, etiolated shoot tissue appears to have very little if any *An1* mRNA compared to light- grown shoots. The presence of message in the roots decouples this light-induced transcription from dependence on chloroplast development.

Using *An1*- specific primers derived from the *An1* cDNA sequence, both qualitative and quantitative measurements of *An1* transcript were made. The primers used were: 5'-TTGCCAAGCTCTGCATCAGCTTGAGTGT-3' as a forward primer, and 5'-GGAAACATGTCTATCGATC-ATATGTTGTGGGGA-3' as a reverse primer. By reverse transcriptase polymerase chain reaction (RT-PCR), using these primers, the distribution of *An1* transcript in maize was determined to include: shoots, roots, silks, pollen, and tassels. Quantitative (Q-)RT-PCR using a competitive template (an *An1* cDNA subclone with a 120 bp λ insert), it was determined that *An1* transcripts accumulate upon exposure to light in maize shoots. Therefore, *An1* transcript accumulation is induced by light. By the same Q-RT-PCR approach *An1* transcript accumulation was shown to be repressed by GA treatment of plants.

EXAMPLE 4

Cloning *An2* by *RT-PCR*

A deletion mutant in maize, designated an1-bz2-6923, produces 20% of the wild-type amount of biosynthetic product of the *An1* gene. This production occurs despite the fact that the deletion mutant totally lacks *An1* transcript and there is no evidence of genomic *An1* DNA. Therefore, it was believed that the *An1* gene has a functional homologue that catalyzes the production of the 20% residual activity. *A priori* this functional homologue could, be but is not necessarily homologous to *An1*. To locate the structural homologue to *An1*, a large number of primers to *An1* were generated and tested by RT-PCR to see if any produced a PCR product using RNA isolated from the deletion mutant. Based on the lack of *An1* DNA in the

deletion mutant, RT-PCR products thus derived were used. One primer pair yielded an RT-PCR product. That primer pair was 5'CTTCGAGATCGCCTTCCCTTCTCTCA-3' (5266) as the forward primer, and 5'-TAGCCCAGCAAATCCCAT-CTTCAGTCCA-3' (5267) as the reverse primer. This primer pair produced a 485 bp product that was subcloned and sequenced. The nucleotide sequence was 82% identical to *An1*. as aligned in FIG. 6 The predicted amino acid level was 82% identical and 91% similar to that of *An1*. This very high per cent of homology suggested that *An2* is a functional duplicate of *An1*.

EXAMPLE 5

Distinguishing *An1 from* An2

In the 485 bp region of interest, *An1* and *An2* each have unique PstI sites which allow the two genes to be distinguished when analyzing PCR products, cDNA libraries, or selecting a colony. The PstI polymorphism was used to screen libraries for the presence of *An2*. The presence of both genes in a maize seedling library resulted in the four band PstI digest pattern shown in FIG. 7. The top and bottom bands are attributable to *An1*, and the middle two bands are attributable to *An2*. The original primers, #5266 and #5267, were paired with primers homologous to "anchor" sequences located at the 5' and 3' ends of a seedling cDNA library that was shown to contain *An1* and *An2*, and entire *An1* and *An2* cDNAs were generated by PCR as two fragments, sized 1.2 and 2.1 kb. Subcloning and transformation of these fragments into competent cells was followed by analysis of plasmid preparations from individual clones. PstI digestion of plasmid preparations revealed *An2* cDNA clones for both the 1.2 and 2.1 fragments.

EXAMPLE 6

Use of Recombinant Genetic Methods to Affect Plant Development

Recombinant genetic methods make use of an isolated DNA molecule encoding a gene product which is necessary to convert GGPP to *ent*-kaurene in the biosynthesis of GA. The isolated DNA molecule is incorporated into a plasmid, such as that shown in FIGURE 5, and transferred into a host plant. The expression of the DNA in the host will generally increase the endogenous levels of GA. The effect will depend on the species and the increment in GA levels. As shown herein, *an* mutations can affect time to maturity.

A strong, constitutive promoter is generally preferred to regulate a gene of the present invention in a host cell. Examples of suitable promoters are ubiquitin and 35S.

Decreasing endogenous GA levels is achieved by introducing an antisense molecule to a gene product of the present invention. Knowledge of the binding domain sequence (FIGURE 2) allows such antisense molecules to be specifically constructed.

Directed mutation is useful to change a phenotypic gene of the present invention so that GA levels are reduced. The effects of reduced GA levels have been described above. Knowledge of a sequence of a maize *An1* and a partial sequence of a maize *An2* gene will facilitate targeted, site specific mutations not only in maize, but in other monocots which as described herein have homologues to *An1* of maize.

Table 2. Kaurene Accumulation in Shoots of Light Grown Maize Seedlings.

	Ent-Kaurene Content (pmoles/gfwt)		Leaf Length(mm)	
Plant	No Treatment	48h Tetcyclacis	2nd Leaf	3rd Leaf
an1-bz2-6923				
Tall	120	1330	42	83
Dwarf	33	209	30	58
an1-891339				
Tall	61	710		
Dwarf	54	216		
d5				
Dwarf	not detected	not detected		
B73	94	1093		

Seedlings were grown in continuous light for six days, at which time mM tetcyclacis (an inhibitor of kaurene metabolism) was applied directly to the shoots. Forty-eight hours later, the shoots of treated and non-treated plants were analyzed for *ent*-kaurene content.

EXAMPLE 7

An1 promoter-GUS Fusion Constructs and Expression

Two thousand bases immediately 5' to the *An1* start of transcription (i.e. the *An1* promoter) have been cloned and sequenced. The sequence is shown in FIGS. 8A and B. This 2kb promoter region was fused to GUS. Transient expression assays on germinating seedlings demonstrated that the *An1* promoter is sufficient for expression of the GUS fusion protein in roots and shoots.

METHODS

Plant Material

A Mu2 tagged *An1* maize family, *an1-891339*, was selected from lines with active Mu elements (lines originated from Pat Schnable, Iowa State University). Additional *An1* alleles used in this study include; *an1bm2* (110D, Maize Genetics Cooperation Stock Center, U.Illinois), *idd*-2286A* and *an1-bz2-6923* (both from G. Neuffer, U.Missouri). *idd*-2286A* is mutated in both the

indeterminate locus (*id*) and the *An1* locus (*d*) but does not appear to be a deletion mutant, as progeny of selfs from this material segregate for *id* and *An1*. Conversely, *an1-bz2-6923* appears to be a deletion mutant. The extent of the deletion is not defined although *Id* (two map units proximal to *An1*) and *Ad* (two map units distal from *Bz2*) are unaffected by the deletion.

Southern Analysis

Total DNA was extracted from leaf tissue by the urea extraction method (Dellaporta *et al.*, 1983). Southern blots were performed as previously described (Johal, 1992) using Duralose-UV membranes (Stratagene). Mu2 probes were synthesized by random priming (Amersham) a gel-eluted internal 650-bp AvaI-BstEII Mu1 fragment isolated from pA/B5 (Chandler, 1986). This internal Mu1 fragment contains regions of homology to Mu2, thus allowing for hybridization to both Mu1 and Mu2 sequences.

Cloning Protocol

The genomic DNA restriction fragment containing the Mu2 element judged to cause the *an1-891339* mutation was electro-eluted following preparative agarose gel electrophoresis of SstI digested total DNA, dialyzed, and concentrated by ethanol precipitation. Precipitated fragments were pre-annealed to SstI restricted arms of the bacteriophage vector lambda sep6/lac5 (Meyerowitz, from Marteinssen, CSH) and packaged using Gigapack Gold (Stratagene). This library was screened for Mu2 containing phage, with the SstI insert of a plaque purified Mu2 containing clone then transferred to the bacteriophage vector Lambda-ZAPII (Stratagene). This insert and other clones used for probing or sequencing were all sub-cloned into the plasmid Bluescript SK+ and maintained in SURE cells (Stratagene).

cDNA Library Screening

Two cDNA libraries, which served as sources for *An1* cDNAs, were prepared from the shoots of 14 day old light grown B73 seedlings, a gift from A. Barkan, University of Oregon (Barkan, 1991) and from whole kernels (30 DAP) of

W22, a gift from Karen Cone, University of Missouri. Sequence data from a 2.8kb *An1* cDNA was generated by Loftstrand Labs Limited.

RNA Preparation and Northern Analysis

Total RNA was prepared as previously described (Chomczynski *et al.*, 1987). PolyA$^+$RNA was enriched using PolyA-Tract System III (Promega) following the manufacturer's protocol. Northerns were run, blotted and probed as previously described (Johal, 1992) using 1.5kb and 1.1kb subclones of *An1* cDNA to generate random primed probes.

Analysis of *ent*-Kaurene and Kaurene Synthetase Activity

Analysis of the *in vivo* accumulation of *ent*-kaurene in light grown maize seedlings was performed. Cell free assays of kaurene synthetase A and B activities were performed using immature siliques from *Arabidopsis* seedlings. (Bensen, 1995).

Production of a Transgenic Plant

A transgenic plant containing a construct having a gene of the present invention can be regenerated from a culture transformed with that same construct, so long as plant species involved is susceptible to regeneration. "Culture" in this context comprehends an aggregate of cells, a callus, or derivatives thereof that are suitable for culture.

A plant is regenerated from a transformed cell or culture, or from an explant, by methods disclosed herein that are known to those of skill in the art. Methods vary according to the plant species. Seed is obtained from the regenerated plant or from a cross between the regenerated plant and a suitable plant of the same species using breeding methods known to those of skill in the art.

Example of Transformation Methods in Maize (May be Modified for Specific Promoters and Structural Genes)

Maize Tapetum Specific Promoter: Stable Transformations Experimental Protocols

Repetition 1,2, and 5;

Goal: Recover transgenic colonies, plants and progeny of maize resistant to Basta/Bialophos and expressing GUS driven by the tapetum specific SGB6g1 promoter

Genotype: 54-68-5 B1-1 (Repetition 1) or
54-68-5 161F3 (Repetition 2)
54-68-5 161F4 (Repetition 5)

Medium: 237 liquid suspension medium for maize
115, callus maintenance medium for maize
115E, callus 5mg/L Basta selection medium
115B, callus 3mg/L Bialaphos selection medium

Tissue Treatment

-Sieve cells through 710um mesh one day after subculture

-Resuspend in 237+3% PEG at 50mg/ml plate density

-Incubate in 3% PEG overnight

-Plate cells, 0.5ml/plate onto glass filters 934-AH atop a Whatman filter moistened with 1ml 237+3% PEG medium

-Transfer cells on glass filter to 115 medium following bombardment

Particle gun bombardment

DuPont helium gum (Repetitions 1 and 5)

650 PSI rupture disks (Repetitions 1 and 5)

DuPont PDS-1000 gun (Repetition 2)

0.230" stopping plates, Acetyl macroprojectiles (Repetition 2)

One bombardment per sample (Repetitions 1 and 5)

Two bombardments per sample (Repetition 2)

Pioneer tungsten modified DNA protocols, specific to each gun

DNA:

DP687+DP610

DP460+DP610

DP1952+DP610

DP2125+DP610

Treatment/Assay following bombardment

-Look for R gene expression 24-48 hours post bombardment

-Transfer samples to 115E (Repetition 1) 48 hours post bombardment. Transfer samples to 115B (Repetition 2 and 5) 7 days post bombardment

-Transfer cells off filters 2 weeks following transfer to selection

-PCR assay colonies for reporter gene prior to plant regeneration

-Maintain samples at 28C in the dark

Method of corn transformation to recover stable transgenic plants

Day-1 Cells placed in liquid media and sieved (710um), 100-200 mg of cells collected on 5.5 cm glass fiber filter over an area of 3.5 cm. Cells transferred to media and incubated media over night.

Day 0 Filter and cells removed from media, dried and bombarded. Filter and cells placed back on media.

Day 5 Cells on filter transferred to selection media (3 mg bialophos).

Day 12 Cells on filter transferred to fresh selection media.

Day 19 Cells scraped form filter and dispersed in 5 ml of selection media containing 0.6% low melting point sea plaque agarose. Cells and media spread over the surface of two 100mm x 15mm plate containing 20 ml of gel-rite solidified media.

Day 40 Putative transformants picked from plate.

Day 61 Plates checked for new colonies.

REFERENCES

The references listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques or compositions employed herein.

Altschul *et al.* (1990). *J. Mol. Biol.* 215:403.

Barendse, G.W.M., Dijkstra, A. and Moore, T.C. (1983). The biosynthesis of the gibberellin precursor *ent*-kaurene in cell-free extracts and the endogenous gibberellins of Japanese morning glory in relation to seed development. *J.Plant Growth Regul.* 2, 165-175.

Barkan, A. and Marteinssen, R.A. (1991). *Proc. Natl. Acad. Sci. USA* 88, 3502.

Beavis, W.D., Grant, D., Albertsen, M. and Fincher, R. (1991). *Theor. Appl. Genet.* 83:141-145.

Bensen, R. J. *et al.* (1995) Cloning and characterization of the An1 gene, *The Plant Cell* 7:75-84.

Buckner, B., Kelson, T.L. and Robertson, D.S. (1990). *The Plant Cell* 2:867-876.

Chandler, V.L. and Walbot, V. (1986). DNA modification of a maize transposable element correlates with a loss of activity. *Proc. Natl. Acad. Sci. USA* 83:1767.

Cheng, P.C., Greyson, R.I. and Walden, D.B. (1983). Organ initiation and the development of unisexual flowers in then tassel and ear of zea mays. *Amer. J. Bot.* 70, 450-462.

Chomczynski, P. and Sacchi, N. (1987). *Anal. Biochem.* 162, 156.

Coolbaugh, R.C. (1985). Sites of gibberellin biosynthesis in pea seedlings. *Plant Physiol.* 78, 655-657.

Dellaporta, S.L., Wood, J.B. and Hicks, J.B. (1983). *Plant Mol. Biol. Rep.* 1, 18.

Duncan, J.D. and West, C.A. (1981). Properties of kaurene synthetase from *Marah macrocarpus* endosperm: evidence for the participation of separate but interacting enzymes. *Plant Physiol.* 68, 1128-1134.

Emerson, R.A. and Emerson, S.E. (1922). Genetic interrelations of two andromonecious types of maize. *Genetics* 7, 203-227.

Facchini, P. and Chappell, J. (1992). Gene family for an elicitor-induced sesquiterpene cyclase in tobacco. *Proc. Natl. Acad. Sci. USA* 89, 11088-11092.

Fujioka, S., Yamane, H., Spray, C.R., Gaskin, P., MacMillain, J., Phinney, B.O. and Takahashi, N. (1988). Qualitative and quantitative analysis of gibberellins in vegetative shoots of normal, *dwarf-1, dwarf-2, dwarf-3,* and *dwarf-5* seedlings of *Zea mays*. *L. Plant Physiol.* 88:1367-1372.

Han, C.d., Coe, Jr., E.H. and Marteinssen, R.A. (1992). *EMBO* 11:4037-4046.

Hedden, P., Phinney, B.O., Heupel, R., Fujii, D., Cohen, H., Gaskin, P., MacMillian, J. and Graebe, J.E. (1982). Hormones of young tassels of *Zea mays*. *Phytochemistry* 21:391-393.

Johal, G.S. and Briggs, S.P. (1992). Reductase activity encoded by the *HM1* disease resistance gene in maize. *Science* 258:985-987.

McCarty, D.R., Carlson, C.B., Stinard, P.S. and Robertson, D.S. (1989). *The Plant Cell* 1:523-532.

McLaughlin, M. and Walbot, V. (1987). *Genetics* 117:771-776.

Marteinssen, R.A., Barkan, A., Freeling, M. and Taylor, W.C. (1989). *EMBOJ* 8:1633-1639.

Matthews, B.F., Wadsworth, G., Gebhardt, J.S. and Wilson, B. (1993). Cloning and expression of genes encoding aspartate aminotransferase in soybean. *Improved Crop and Plant Products Through Biotechnology*, Abs. X1-324, pp. 105.

Metzger, J.D. and Zeevart, J.A.D. (1980). Effect of photoperiod on the levels of endogenous gibberellins in spinach as measured by combined gas chromatography-selected ion current monitoring. *Plant Physiol.* 66, 844-846.

Metzger, J.D. and Zeevart, J.A.D. (1982). Photoperiodic control of gibberellin metabolism in spinach. *Plant Physiol.* 69, 287-291.

O'Reilly, C.O., Shepherd, N.S., Pereira, A., Schwartz-Summer, Z., Bertram, I., Robertson, D.S., Peterson, P.A. and Saedler, H. (1985). *EMBOJ* 4:877-882.

Pasternak *et al.* (1993). Sequence Similarity Searches, Multiple Sequence Alignments, and Molecular Tree Building in *Methods in Plant Molecular Biology and Biotechnology* Glick *et al.* (eds.), (CRC Press), pp. 251-267.

Rood, S.B., Buzzell, R.I., Mauder, L.N., Pearce, D. and Pharis, R.P. (1988). *Science* 241:1216-1218.

Simcox, P.D., Dennis, D.T. and West, C.A. (1975). Kaurene synthetase from plastids of developing plant tissues. *Biochem. Biophys. Res. Comm.* 66:166-172.

Sun, Goodman and Ausubel (1992). *The Plant Cell* 4:119-128.

Suzuki, Y., Yamane, H., Spray, C.R., Gaskin, P., MacMillian, J. and Phinney, B.O. (1992). Metabolism of *ent*-kaurene to gibberellin A_{12}-aldehyde in young shoots of normal maize. *Plant Physiol.* 98, 602-610.

Walbot, V. and Warren, C. (1988). Regulation of Mu element copy number in maize lines with an active or inactive transposable element system. *Mol. Gen. Genet.* 211:27-34.

Walbot, V. (1992). Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. Ann. Rev. Plant Physiol. 43:49-82.

PCT patent application WO/9316096.

WHAT IS CLAIMED IS:

1. An isolated DNA molecule capable of hybridizing with a monocot nucleotide sequence under conditions of high stringency, said molecule encoding a product affecting the conversion of GGPP to *ent*-kaurene in the biosynthesis of gibberellic acid.

2. The DNA molecule of claim 1 capable of hybridizing to a nucleotide sequence according to the *An1* sequence of FIGURE 3 under conditions of high stringency.

3. The isolated DNA molecule of claim 1 having the nucleotide sequence of FIGURE 3.

4. The isolated DNA molecule of claim 1, having a partial sequence of FIGURE 6.

5. The isolated DNA molecule of claim 1 having a mutation altering the product affecting the conversion of GGPP to *ent*- kaurene.

6. An *An1* gene cloned from maize.

7. An *An2* gene cloned from maize.

8. An expression vector comprising the DNA molecule of claim 1 and a promoter controlling expression of the molecule.

9. The expression vector of claim 8 wherein the promoter is according to FIGURE 8A and B.

10. A polypeptide encoded by the expression vector of claim 8.

11. The polypeptide of claim 10 having an *An1* amino acid sequence according to according to FIGURE 2.

12. A method for altering the level of gibberellic acid endogenous to a plant of a first species, comprising transferring an isolated DNA molecule capable of encoding a product used for the conversion of GGPP to *ent*-kaurene to a host cell from which the plant is regenerated.

13. The method of claim 11, wherein said transferred DNA molecule affects 12, the endogenous GA level such that the time of maturity of said plant is altered relative to the norm for said first species.

14. A method for altering the level of gibberellic acid endogenous to a plant of a first monocot species, said method comprising constructing an antisense molecule to the isolated DNA molecule of claim 1 and delivering the antisense molecule to the plant in sufficient amounts and at suitable times in development to decrease Ga levels.

FIG. 1

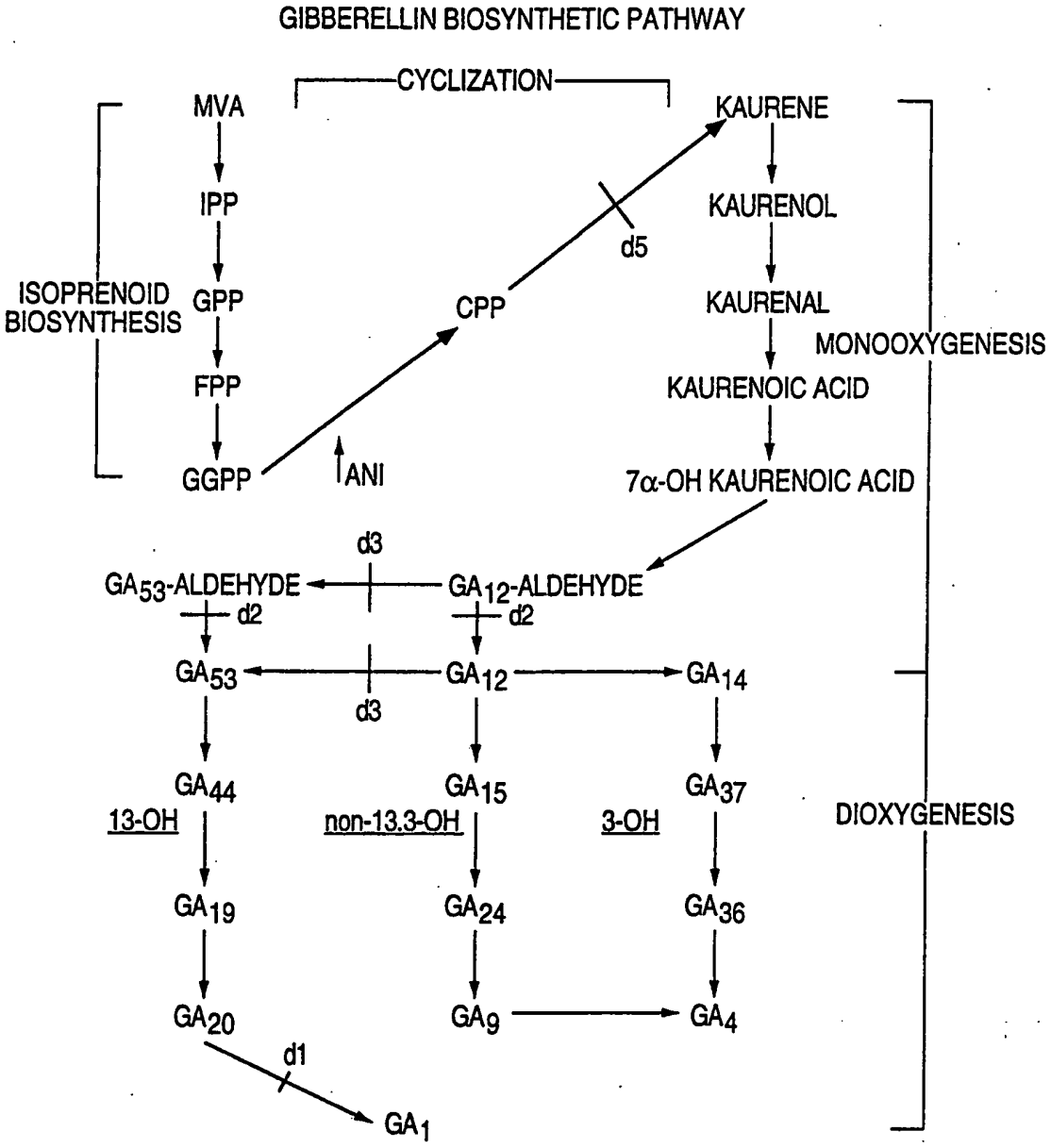

GIBBERELLIN BIOSYNTHETIC PATHWAY
CYCLIZATION
MVA
IPP
GPP
FPP
GGPP
ISOPRENOID BIOSYNTHESIS
ANI
CPP
d5
KAURENE
KAURENOL
KAURENAL
KAURENOIC ACID
7α-OH KAURENOIC ACID
MONOOXYGENESIS
d3
GA53-ALDEHYDE
GA12-ALDEHYDE
d2
d2
GA53
GA12
GA14
d3
GA44
GA15
GA37
13-OH
non-13.3-OH
3-OH
DIOXYGENESIS
GA19
GA24
GA36
GA20
GA9
GA4
d1
GA1

FIG. 1B-1

FIG. 1B-2

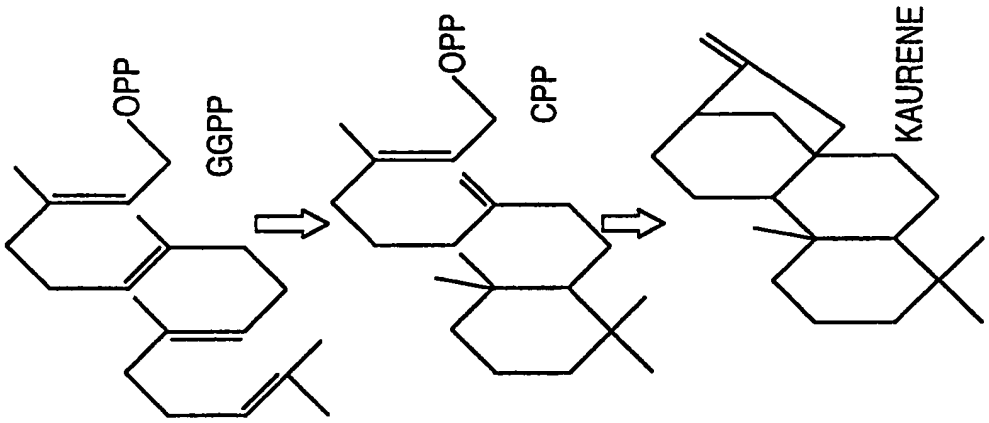


FIG. 1B-3

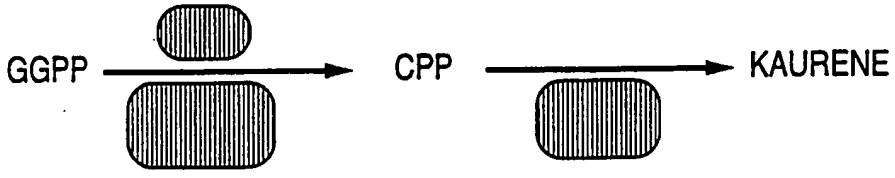


FIG. 2A

```
  1 .........MPYPHPYPWQSSRRRRRRG..RDGAPRQPQAPRVVERAAAGP  41
  1 MSLQYHVLNSIPSTTFLSSTKTTISSSFLTISGSPLNVAR...DKSRSGS  47

 42 GHATTTQQPDNVSSAKVFQTSRVETESKLRNGRKPQDLEDEHQAEEAELQ  91
 48 IHCSKLRTQEYINSQEVQHDLPLIHEWQQLQGEDAPQISVG..SNSNAFK  95

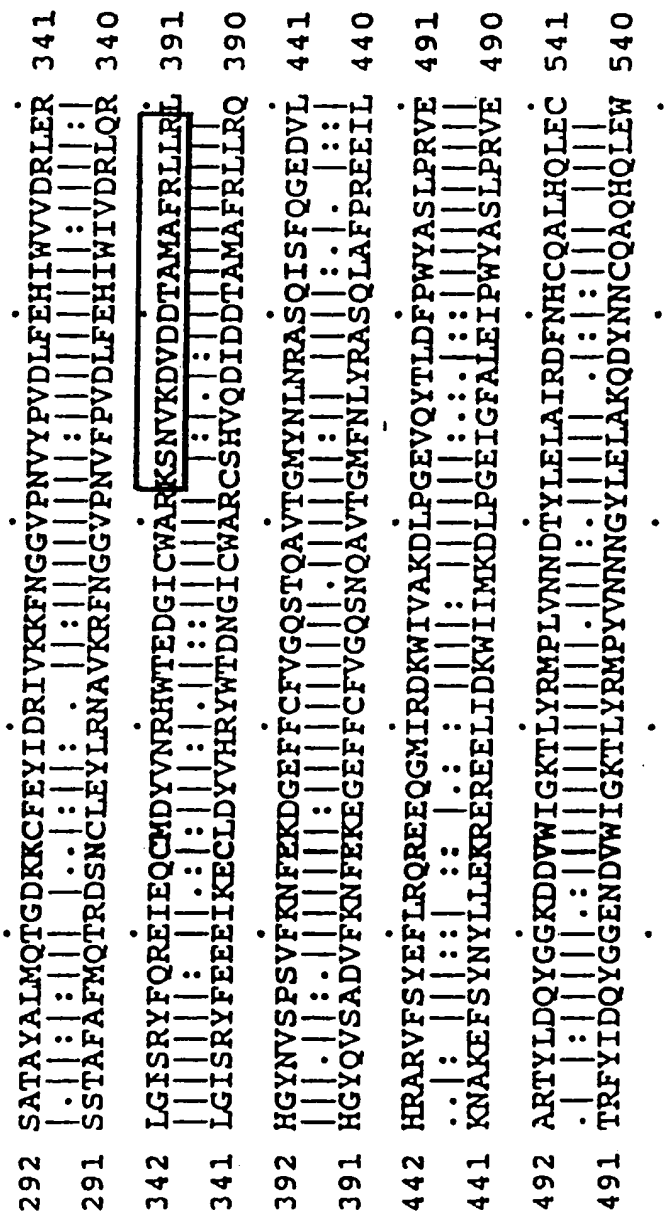
 92 PLIDQVRAMLRSMNDGDTSASAYDTAWVAMVPKVGGDGGAQPQFPATVRW  141
 96 EAVKSVKTILRNLTDGEITISAYDTAWVALI....DAGDKTPAFPSAVKW  141

142 IVDHQLPDGSWGDSALFSAYDRMINTLACVVALTKWSLEPARCEAGLSFL  191
142 IAENQLSDGSWGDAYLFSYHDRLINTLACVVALRSWNLFPHQCNKGITFF  191

192 HENMWRLAEEEAESMPIGFEIAFPSLIQTARDLGVVDFPYGHPALQSIYA  241
192 RENIGKLEDENDEHMPIGFEVAFPSLLEIARGIN.IDVPYDSPVLKDIYA  240

242 NREVKLKRIPRDMMHRVPTSILHSLEGMPDLDWPRLLNLQSCDGSFLFSP  291
241 KKELKLTRIPKEIMHKIPTTLLHSLEGMRDLDWEKLLKLQSQDGSFLFSP  290
```

FIG. 2B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291 SSTAFAFMQTRDSNCLEYLRNAVKRFNGGVPNVFPVDLFEHIWIVDRLQR 340
342 LGISRYFQREIEQCMDYVNRHWTEDGICWARKSNVKDVDDTAMAFRLLRL 391
341 LGISRYFEEEIKECLDYVHRYWTDNGICWARCSHVQDIDDTAMAFRLLRQ 390
392 HGYNVSPSVFKNFEKDGEFFCFVGQSTQAVTGMYNLNRASQISFQGEDVL 441
391 HGYQVSADVFKNFEKEGEFFCFVGQSNQAVTGMFNLYRASQLAFPREEIL 440
442 HRARVFSYEFLRQREEQGMIRDKWIVAKDLPGEVQYTLDFPWYASLPRVE 491
441 KNAKEFSYNYLLEKREREFELIDKWIIMKDLPGEIGFALEIPWYASLPRVE 490
492 ARTYLDQYGGKDDVWIGKTLYRMPLVNNDTYLELAIRDFNHCQALHQLEC 541
491 TRFYIDQYGGENDVWIGKTLYRMPYVNNNGYLELAKQDYNNCQAQHQLEW 540

FIG. 2C

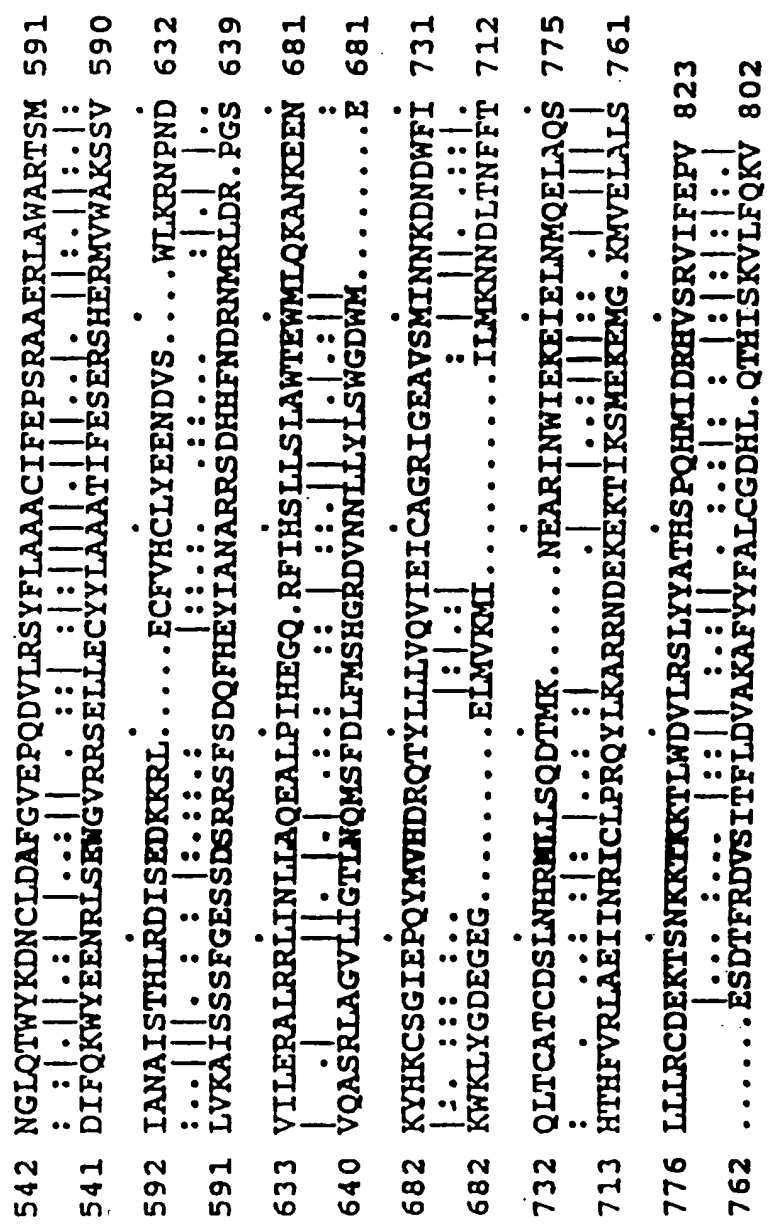


FIG. 3A

```
GAATTCCGCT AGCTCTTGCT TTGTTGTGTG TCCTGATGGT CGAGTTCCTC ACCGTGCTTT    60
TGCTTTTCTG CTTTCACTTG CCTGCAGCTG CAGCTCGTCA ATCAGGTCCA TGCCGTATCC   120
GCATCCGTAT CCGTGGCAAA GCAGCAGGAG GAGGAGGAGG AGGCGCGGGC GCGACGGGGC   180
CCCGCGGCAG CCTCAGGCTC GCCGGGTGGT GGAGCGCGCA GCAGCAGGCC CCGGCCACGC   240
GACGACAACG CAGCAGCCCG ACAACGTCTC CAGTGCTAAA GTGTTCCAGA CCAGCCGTGT   300
GGAAACCGAG TCGAAATTGC GAAATGGCAG GAAACCACAA GACCTTGAGG ATGAGCACCA   360
GGCTGAGGAG GCAGAGCTGC AGCCACTTAT CGACCAGGTG AGGGCGATGC TACGGTCGAT   420
GAACGACGGG GATACCAGCG CCTCGGCGTA CGACACGGCG TGGGTGGCGA TGGTGCCGAA   480
GGTGGGCGGC GACGGCGGCG CCCAGCCCCA GTTCCCGGCC ACCGTGCGCT GGATCGTGGA   540
CCACCAGCTG CCCGACGGCT CCTGGGGCGA CTCGGCCCTG TTCTCCGCCT ACGACCGCAT   600
GATCAACACC CTCGCCTGCG TCGTCGCGCT GACCAAGTGG TCGCTGGAGC CCGCGAGGTG   660
CGAGGCGGGG CTCTCGTTCC TGCACGAGAA CATGTGGAGG CTAGCGGAGG AGGAGGCGGA   720
GTCGATGCCC ATCGGCTTCG AGATCGCCTT CCCTTCTCTC ATCCAGACGG CTAGGGACCT   780
GGGCGTCGTC GACTTCCCGT ACGGACACCC GGCGCTGCAG AGCATATACG CCAACAGGGA   840
AGTCAAGCTG AAGCGGATCC CAAGGGACAT GATGCACAGG GTCCCGACGT CCATCCTGCA   900
CAGCCTTGAA GGGATGCCTG ACCTGGACTG GCCGAGGCTT CTGAACCTCC AGTCCTGCGA   960
CGGCTCCTTC TTGTTCTCTC CTTCGGCTAC CGCTTACGCG CTGATGCAAA CCGGTGACAA  1020
```

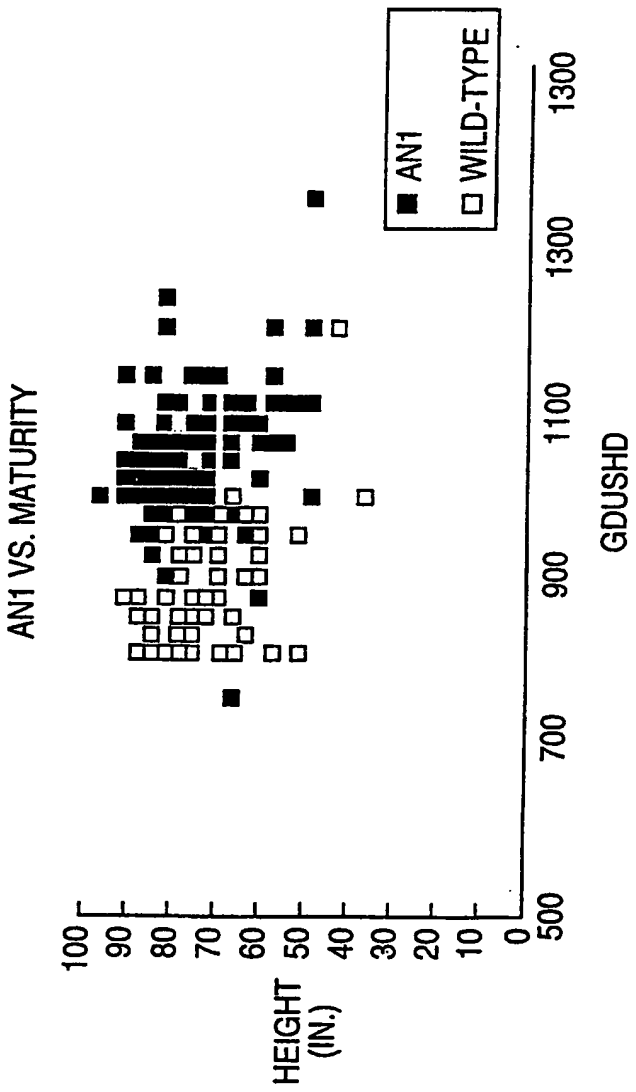
FIG. 3B

```
GAAGTGCTTC GAATACATCG ACAGGATTGT CAAAAAATTC AACGGGGGAG TCCCCAATGT   1080
TTATCCGGTC GATCTTTTCG AGCACATCTG GGTTGTGGAT CGGTTGGAGC GACTCGGGAT   1140
CTCCCGCTAC TTCCAACGAG AGATTGAGCA GTGCATGGAC TATGTGAACA GGCACTGGAC   1200
TGAAGATGGG ATTTGCTGGG CTAGGAAATC CAATGTGAAG GATGTGGATG ACACAGCTAT   1260
GGCTTTCCGA CTACTAAGGC TACATGGATA CAATGTCTCT CCAAGTGTGT TTAAGAACTT   1320
TGAGAAAGAT GGAGAGTTCT TTTGTTTTGT GGGCCAATCG ACTCAAGCCG TCACTGGGAT   1380
GTATAACCTC AACAGAGCCT CTCAGATAAG TTTTCAAGGA GAGGATGTAT TGCATCGTGC   1440
TAGGGTTTTC TCGTATGAGT TTCTGAGACA GAGAGAAGAA CAAGGCATGA TCCGTGATAA   1500
ATGGATCGTT GCCAAGGATC TACCTGGCGA GGTGCAATAT ACACTAGACT TCCCTTGGTA   1560
TGCAAGCTTG CCTCGTGTAG AGGCAAGAAC CTATCTAGAT CAATATGGTG GTAAAGATGA   1620
CGTTTGGATT GGAAAGACAC TCTACAGGAT GCCTCTTGTG AATAACGACA CATATCTAGA   1680
GTTGGCAATA AGGGATTTCA ACCATTGCCA AGCTCTGCAT CAGCTTGAGT GTAATGGGCT   1740
GCAAACGTGG TACAAGGATA ATTGCCTTGA CGCTTTTGGA GTAGAACCAC AAGATGTTTT   1800
AAGATCTTAC TTTTTAGCTG CTGCTTGCAT TTTTGAACCT AGCCGTGCTG CTGAGCGGCT   1860
TGCATGGGCT AGAACGTCAA TGATTGCCAA TGCCATTTCT ACACATCTTC GTGACATTTC   1920
GGAAGACAAG AAGAGATTGG AATGTTTCGT GCACTGTCTC TATGAAGAAA ACGATGTATC   1980
```

FIG. 3C

```
ATGGCTTAAA CGAAATCCTA ATGATGTTAT TCTTGAGAGG GCACTTCGAA GATTAATTAA   2040
CTTATTAGCA CAAGAAGCAT TGCCAATTCA TGAAGGACAA AGATTCATAC ACAGTCTATT   2100
GAGTCTTGCA TGGACCGAAT GGATGTTGCA AAAGGCAAAT AAAGAAGAAA ACAAATATCA   2160
CAAATGCAGT GGTATAGAAC CACAATACAT GGTTCATGAT AGGCAAACAT ACTTACTTTT   2220
AGTTCAGGTT ATTGAGATTT GTGCTGGACG AATTGGTGAG GCTGTGTCAA TGATAAACAA   2280
CAAGGATAAT GATTGGTTTA TTCAACTCAC ATGTGCTACT TGTGACAGTC TTAACCATAG   2340
GATGTTACTG TCCCAGGATA CTATGAAGAA TGAAGCAAGA ATAAATTGGA TTGAGAAGGA   2400
AATCGAGTTG AATATGCAAG AGCTTGCTCA ATCTCTCCTT TTGAGATGTG ATGAGAAAAC   2460
TAGCAATAAG AAGACCAAGA AAACCTTATG GGATGTCCTA AGAAGTTTAT ACTATGCTAC   2520
TCATTCCCCA CAACATATGA TCGATAGACA TGTTTCCAGA GTTATCTTTG AGCCTGTTTA   2580
AAAATGTTTA AGTGGTAGAC CATTATGTTA GGTGTAAATG TGTACATAAA AGTTATCATA   2640
AGGAGTAATG GTAGCAGAAG CATGCAGTTG TAAGTTTATT TGTTGCTTAG AATAGAAATT   2700
AGTGTAGCTA TAATATCAAG AATGTTCCTA TATAAGTAAT CATATTATGG ATAGAGGTGT   2760
TCATATGAAT AATAAAAAGG AATC                                          2784
```

FIG. 4


AN1 VS. MATURITY
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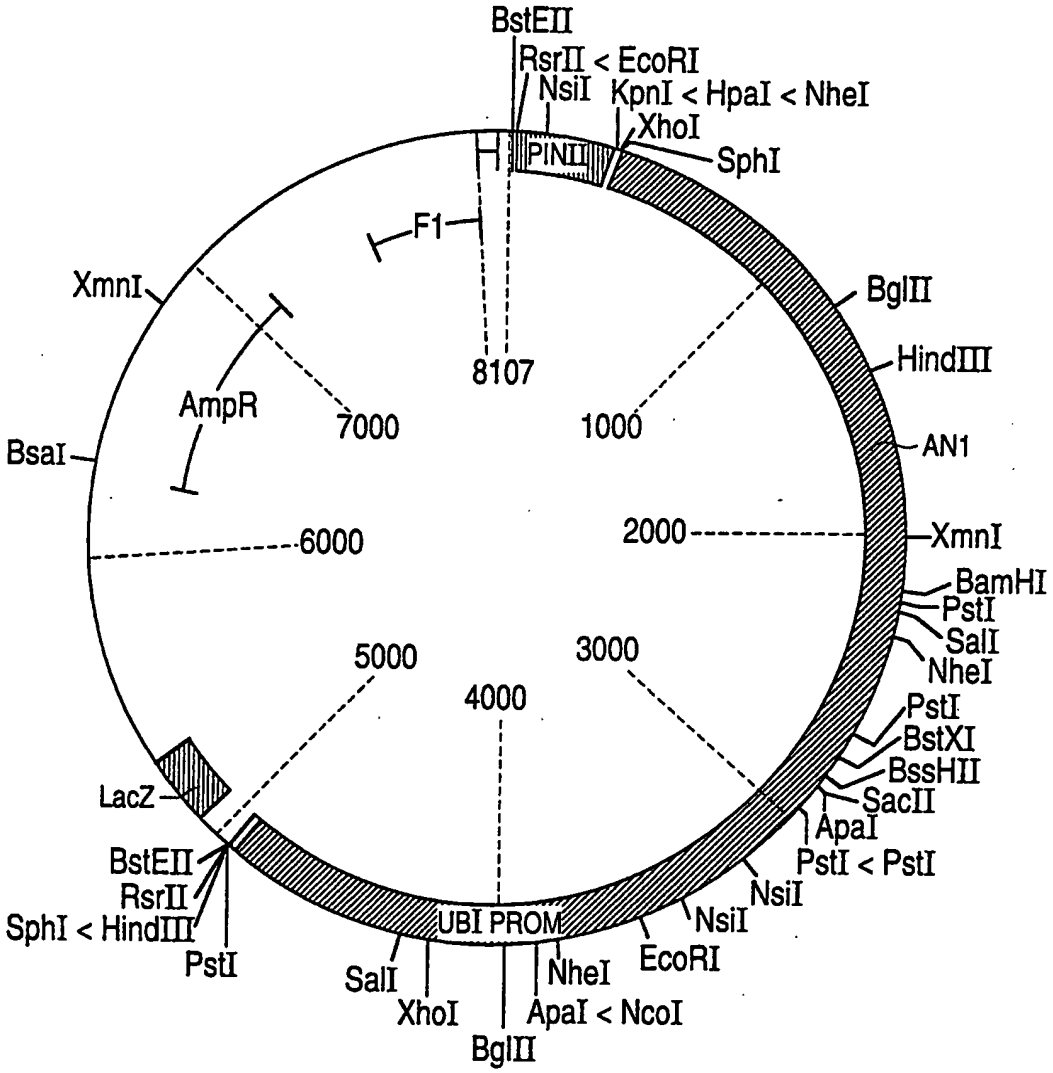
WILD-TYPE
HEIGHT (IN.)
100
90
80
70
60
50
40
30
20
10
0
500
700
900
1100
1300
1300
GDUSHD

FIG. 5


BstEII
RsrII < EcoRI
NsiI
KpnI < HpaI < NheI
XhoI
SphI
PINII
F1
8107
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HindIII
AN1
XmnI
BamHI
PstI
SalI
NheI
PstI
BstXI
BssHII
SacII
ApaI
PstI < PstI
NsiI
NsiI
EcoRI
NheI
ApaI < NcoI
BglII
SalI
XhoI
UBI PROM
BstEII
RsrII
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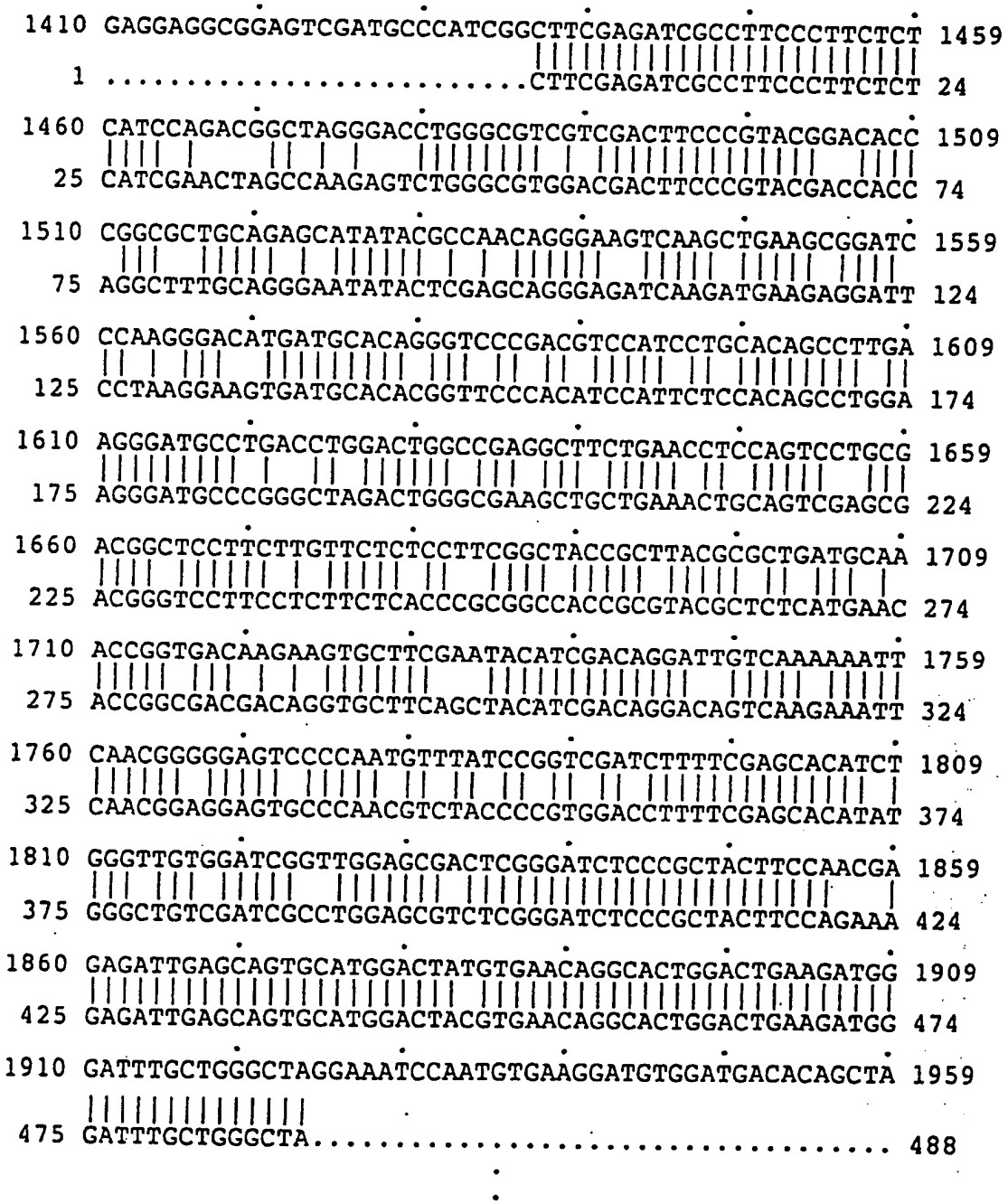
1000
2000
3000
4000
5000
6000
7000

FIG. 6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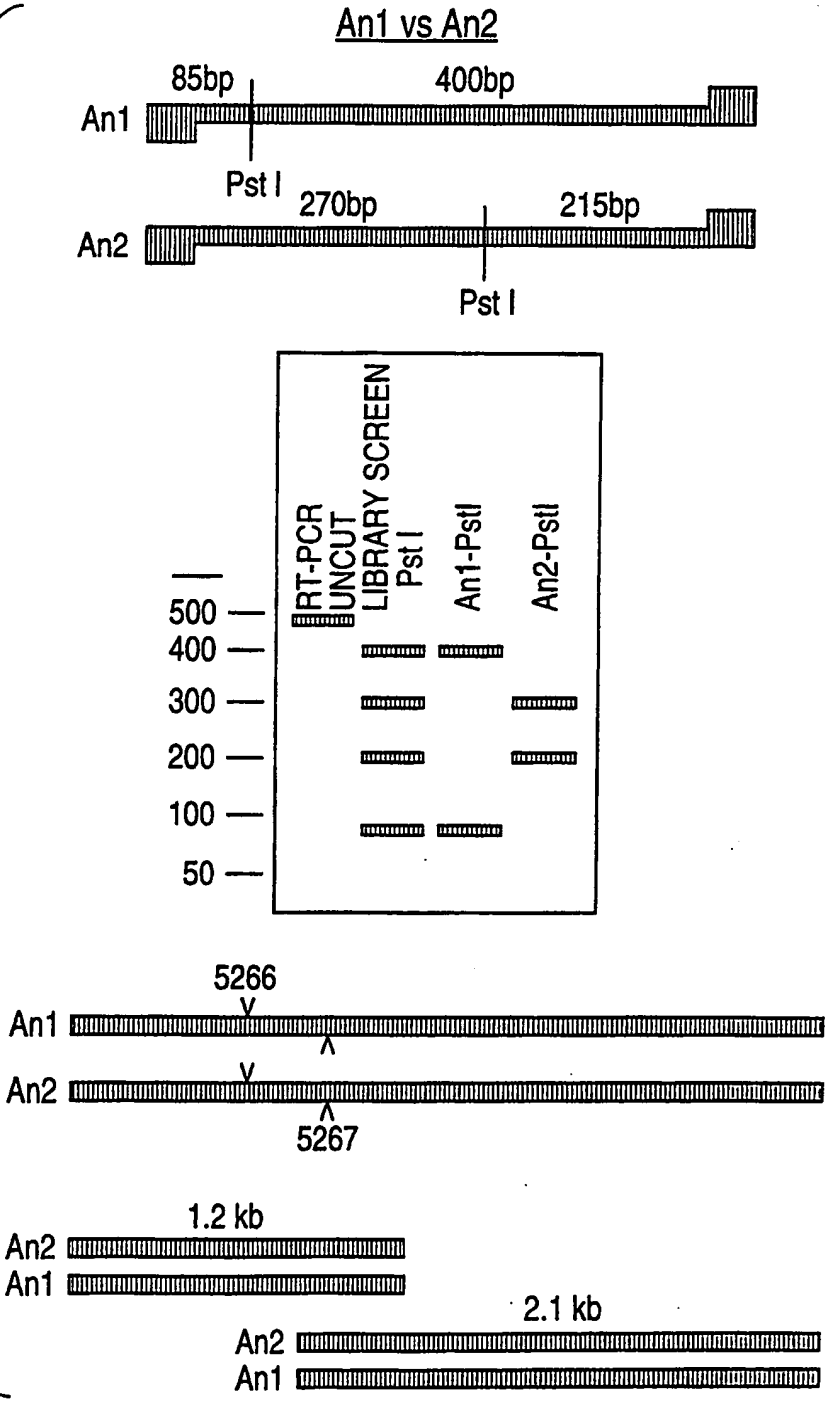
1 .....CTTCGAGATCGCCTTCCCTTCTCT 24
1460 CATCCAGACGGCTAGGGACCTGGGCGTCGTCGACTTCCCGTACGGACACC 1509
25 CATCGAACTAGCCAAGAGTCTGGGCGTGGACGACTTCCCGTACGACCACC 74
1510 CGGCGCTGCAGAGCATATACGCCAACAGGGAAGTCAAGCTGAAGCGGATC 1559
75 AGGCTTTGCAGGGAATATACTCGAGCAGGGAGATCAAGATGAAGAGGATT 124
1560 CCAAGGGACATGATGCACAGGGTCCCGACGTCCATCCTGCACAGCCTTGA 1609
125 CCTAAGGAAGTGATGCACACGGTTCCCACATCCATTCTCCACAGCCTGGA 174
1610 AGGGATGCCTGACCTGGACTGGCCGAGGCTTCTGAACCTCCAGTCCTGCG 1659
175 AGGGATGCCCGGGCTAGACTGGGCGAAGCTGCTGAAACTGCAGTCGAGCG 224
1660 ACGGCTCCTTCTTGTTCTCTCCTTCGGCTACCGCTTACGCGCTGATGCAA 1709
225 ACGGGTCCTTCCTCTTCTCACCCGCGGCCACCGCGTACGCTCTCATGAAC 274
1710 ACCGGTGACAAGAAGTGCTTCGAATACATCGACAGGATTGTCAAAAAATT 1759
275 ACCGGCGACGACAGGTGCTTCAGCTACATCGACAGGACAGTCAAGAAATT 324
1760 CAACGGGGGAGTCCCCAATGTTTATCCGGTCGATCTTTTCGAGCACATCT 1809
325 CAACGGAGGAGTGCCCAACGTCTACCCCGTGGACCTTTTCGAGCACATAT 374
1810 GGGTTGTGGATCGGTTGGAGCGACTCGGGATCTCCCGCTACTTCCAACGA 1859
375 GGGCTGTCGATCGCCTGGAGCGTCTCGGGATCTCCCGCTACTTCCAGAAA 424
1860 GAGATTGAGCAGTGCATGGACTATGTGAACAGGCACTGGACTGAAGATGG 1909
425 GAGATTGAGCAGTGCATGGACTACGTGAACAGGCACTGGACTGAAGATGG 474
1910 GATTTGCTGGGCTAGGAAATCCAATGTGAAGGATGTGGATGACACAGCTA 1959
475 GATTTGCTGGGCTA..... 488

FIG. 7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400bp
An1
Pst I
270bp
215bp
An2
Pst I
RT-PCR
UNCUT
LIBRARY SCREEN
Pst I
An1-PstI
An2-PstI
500
400
300
200
100
50
5266
An1
An2
5267
1.2 kb
An2
An1
2.1 kb
An2
An1

FIG. 8A

```
                                                  10                  20                  30                  40                  50                  60
GAATTCTTTT AAAAAAATAA CGTCTGTGCC TCACAGTAGC TGTAGGAGTA CGGTGTTGCA
                   70                  80                  90                 100                 110                 120
TCTCGCGCTT CAATTCGGTC GACAGCGGCT CCTGTGGTCT CCAGCCTTTA CCGCTTTGGC
                  130                 140                 150                 160                 170                 180
AACTGGTTCT CTTTCAATCA GGTCCATGCC GTATCTTGCT AAAGCTAAAA ATAAATGCGT
                  190                 200                 210                 220                 230                 240
TCACGGGAAC CCCGTGTGTC CGTGTTTCTG CTCTGCTGCA TGCAGCTCTC GCCTTTATTT
                  250                 260                 270                 280                 290                 300
TTTCTTTCCA TCAAAAGCAA CCGATGACCA ACGGCCTTAC ACAGTCTGTC GAGAACTCGA
                  310                 320                 330                 340                 350                 360
GATTCTCCAT CCCCCCAAAT GAAAACGAGG TCGTAAATCT CGCTTCACGT CGGTAAGTCT
                  370                 380                 390                 400                 410                 420
AAAAAATCTT AAATTTAACT CAACTTGTTA AAGATATTGT CAACATTTAG ATGTTTAGTT
                  430                 440                 450                 460                 470                 480
AAATTTACTA TAAAAAAAGT ATCTTAAGAT ACGTCCATTC AGTAAAAAAA AAACAAAAAG
                  490                 500                 510                 520                 530                 540
AAAAACTTTA ACTATTCGAC AAACGAGATT TTTTTTTCCC GGGTTGCCGT CCCACTGCAC
                  550                 560                 570                 580                 590                 600
GGACTAGTTG CCAGGCATCT TCCCTGAAAC AAAAAGGAAA ACACCAGTTC TAGGCACGTA
                  610                 620                 630                 640                 650                 660
CGTACTACGC TTACGTGTAT ATAAATATTT ATCGGCTGTG GACAGATTAT AGAGGGCATC
                  670                 680                 690                 700                 710                 720
TTGTTGCGAC GGGGCGACGA ATGTCCGTCT CCAGCCACCC GTCCGTTCGC AAATCACGTA
                  730                 740                 750                 760                 770                 780
CAAAGCTACT TTGTGTTAGC AGAAACACCA TAAAAAACAA AATAGATCAA CTAACAAATC
                  790                 800                 810                 820                 830                 840
TGGAAGCACG ACACATAAAT TTACTTAAAA ACCTTTCAAT GAGAAAGGAA AAATTATGAG
                  850                 860                 870                 880                 890                 900
CACCAGCCAC TTGATGTGAA GTGTTTATGT AACGTCGTTC GTAGACGGCG GCTTACAAGA
                  910                 920                 930                 940                 950                 960
GAAGTAAAAA GACGACGTGC GATAAATTCT AATTAGGTTC ATTAATATAT ACCTAAGTTT
                  970                 980                 990                1000                1010                1020
TTGCACGATG GGTATCCACT CTGCTCGCTA ATATTGTCTC TATATTCAGA ATTTGGATCA
                 1030                1040                1050                1060                1070                1080
CAAACTTAAT AGTTTGGTTG GTTATGTATA CAGTATACTC ACGCACCAAC GCACGCACGC
                 1090                1100                1110                1120                1130                1140
CGCGAGAAGG ATTAGCGAAA CCCTGGTGGT TTTTTGTCCA CTAGCCGGTG CGTCCCCGCG
                 1150                1160                1170                1180                1190                1200
CGGGAATCAT TCGGGCCTCC CTCTCTACTC TGCTCCCAGC TACTAGTCCC TCACTCACTT
                 1210                1220                1230                1240                1250                1260
CTCTCAGACT TGTGTGTCTC GTCCTATATA TATATACACA CGCTACGCTA TAGCTGCTCA
```

FIG. 8B-1

```
      1270       1280       1290       1300       1310       1320
CACACATAGT ATCGTCGTCT CTCTCTCCTT CCCACCAACA ACGCACGCGT CCAAGAAGGA
      1330       1340       1350       1360       1370       1380
AATGATTAGT AGCAGCCATA CTTGCTCCGC CTATTAATAG CCAGCGCCCC CGTCTTGTTT
      1390       1400       1410       1420       1430       1440
TGCTCTCTTC TGTTCTGTTT TGCCCTAGAT TAGCGGCGGC GTTTTGGCCT CGCGCGAACG
      1450       1460       1470       1480       1490       1500
TCTCGTCCTT GCCGTCTCGC GCGCGTGCGT ACCTGCCTGC ATTGCGATTT CCAATTATTA
      1510       1520       1530       1540       1550       1560
CGATCGCGCG GCGGCGGCGG CCGCGGCATG AAGCTCCTCT CGCCGGCGGC CGCACCGTCG
      1570       1580       1590       1600       1610       1620
TCCTCGCCGT TGTTCCCTCC TCGCATCGTC GAAGGTACGT CTACACCGTC GTCAGCAGCT
      1630       1640       1650       1660       1670       1680
GCTACCTCCG CGGCGCCGGC CAGCCGAGGT TCCATGATGC CTATCTATCT ATGTATAGTA
      1690       1700       1710       1720       1730       1740
CGTATATGGC GCCGCGCCAG GCCCTTGCCC TTGTCGTCTG CCTGCATGCC TACTACTACA
```

FIG. 8B-2

```
      1750        1760        1770        1780        1790        1800
AGCTACTTCC AAATTTCGCA TTGTCCTCCG CGCTACACGG CCGGTGGGCA ATCAGACAAA
      1810        1820        1830        1840        1850        1860
GAAACAAACG TGTAAGCAAG ATGAAAAATT GTATTTTTGG GTTCGGACAA GCAAGTCGTC
      1870        1880        1890        1900        1910        1920
GTCGTCGTCT TACGGTAGCC ACACACACAG GCAGATGGCC AATCAGACAA AGAAACAAAC
      1930        1940        1950        1960        1970        1980
ATAAGCAAGA TGGAGAGACC CAGGCAGGCA CTCAGGCGCT GCTGCTGCTA ATGCTAGCTC
      1990        2000        2010        2020        2030        2040
TTGCTTTGTT GTCTCTCCTG ATGGTCGAGT TCCTCACCGC TGCTTTTGCT TTTCTGCTTT
      2050        2060        2070        2080        2090        2100
CACTTGCCTG CAGCTGCAGC TCGTCAATCA GGTCCATGCC GTATCCGCAT CCGTATCCGT
      2110        2120        2130        2140        2150        2160
GGCAAAGCAG CAGCAGCACG ACGAGGAGGA GCCCCGGGCG CGACGGGGCC CCGCGGCAGC
                    |G|
      2170        2180        2190        2200        2210        2220
CTCAGGCTCG CCGGGTGGTG GAGAGCGCAG CAGCAGGCCC CGGCCACGGC GACGACAACG
                                                        |G|
      2230        2240        2250        2260        2270        2280
CAGCAGCCTG ACAACGTCTC CAGTGCTAAA GGTGCTAGCT TGCTCGTTAT ATTTGATTTG
                              |G |R | K |
      2290        2300        2310        2320        2330        2340
ACTAGTCTCA TCATCCACCC CCCAGTCACG TACACAGATG CTCTCTCTCT CTCTCTCTCT
      2350        2360        2370        2380        2390        2400
CGAATTCATG AGCGAACCAA ACACTCAGAC AGATGCTGCC GTGCTGCAGT NCGCCCGTAG
      2410        2420        2430        2440        2450        2460
CAGCACAGAC ACTCTGCCCC ACACACCTGC GCTTGTCGCT TCCCCTCTTG CTATATCTCC
      2470        2480        2490        2500        2510        2520
TGCTGCTTTT GCTAAAGCCG GAAACCAAAA AGAAAGTTGA GCTTTTCGTC ACAATTTTGC
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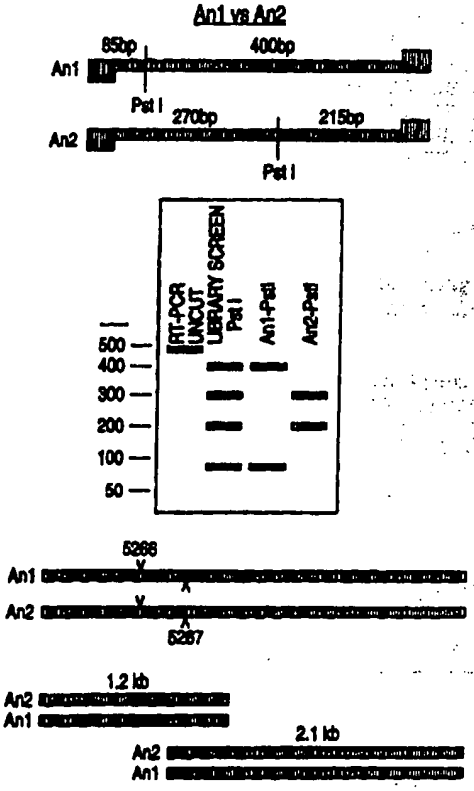
(54) Title: PLANT GENES AFFECTING GIBBERELLIC ACID BIOSYNTHESIS

(57) Abstract

Genes controllong gibberellic biosynthesis are used in genetic engineering to alter plant development. Alterations in the nature or quantity of products of the genes affects plant development. A family of *An* genes in monocots encodes a cyclase involved in the early steps of gibberellic acid (GA) biosynthesis. Members of the family are identified in wheat, barley, sorghum and maize. Two members of the family, the genes *An1* and *An2*, are identified in maize. The *An1* gene is cloned and the function of the gene is characterized. *An2* is isolated and identified by homology to *An1*. Using recombinant genetic technology, GA levels are manipulated. Changes in GA levels alter monocot plant phenotypes, for example, increasing or decreasing height and fertility.



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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 95/07118

A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT PHYSIOL., vol. 102 (Suppl.) (1993), page 8, abstract no. 33, B. BENSEN ET AL.; 'Cloning gibberellin biosynthetic genes from maize' see abstract. & Joint Annual Meeting of the American Soc. of Plant Physiologists and the Canadian Soc. of Plant Physiologists, Minneapolis, Minn., USA, July 31-August 4, 1993. --- -/--	1-3,5,6, 8,10-14

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Inter nal Application No
PCT/US 95/07118

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT PHYSIOL., vol. 99 (Suppl.) (1992), page 19, abstract no. 111, R. BENSEN ET AL.; 'Cloning gibberellic acid biosynthetic genes from maize ...' see abstract. & Annual Meeting of the American Society of Plant Physiologists, Pittsburgh, Penn., USA, August 1-5, 1992.	1-3,5,6, 8,10-14
X	WO-A-93 16096 (THE GENERAL HOSPITAL CORPORATION) 19 August 1993 cited in the application see Examples 1 and 2 and Claims.	1,2,5,8, 10-14
P,X	THE PLANT CELL, vol. 7, 1995 pages 75-84, R.J. BENSEN ET AL.; 'Cloning and characterisation of the maize An1 gene' cited in the application see the whole document.	1-14

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ional Application No
PCT/US 95/07118

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9316096	19-08-93	AU-B-	3659493	03-09-93
		EP-A-	0626971	07-12-94
		HU-A-	69797	28-09-95
		JP-T-	7503850	27-04-95